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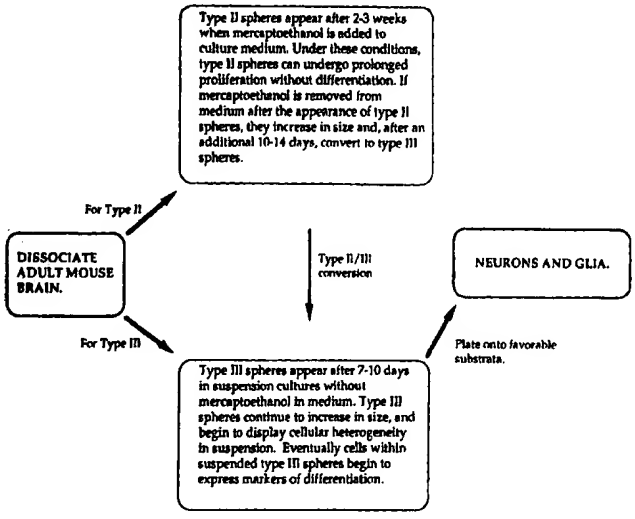
(54) Title: ISOLATED MAMMALIAN NEURAL STEM CELLS, METHODS OF MAKING SUCH CELLS, AND METHODS OF USING SUCH CELLS



(57) Abstract: Stem/progenitor cells in the developing and adult CNS can form multipotent clones or neurospheres when cultured in the presence of growth factors. Multipotent stem/progenitor cells have been isolated and cultivated from adult human brain specimens following protracted postmortem intervals. Astrocytes cultured as monolayers formed neurospheres in cultures derived from many different CNS regions, including cerebral cortex, subependymal zone, and cerebellar cortex; furthermore, astrocytes possessed this ability even after multiple passages. Several cell phenotype immunomarkers for astrocytes and neurons that were generated from neurospheres contained cells that were immunoreactive for these markers. Neurospheres have been shown to have attributes of stem/progenitor cells under particular tissue culture conditions.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

1.0 BACKGROUND OF THE INVENTION

This application is a continuation-in-part of U.S. Application Serial No. 09/402,227 filed October 1, 1999 which is a continuation of PCT/US98/00366 filed January 7, 1998 which is a continuation-in-part of and takes priority from U.S. Provisional Application Serial No. 60/034,910 filed January 7, 1997 the entire contents of each of which is specifically incorporated herein by reference in its entirety without disclaimer.

The United States government has rights to use of the present invention with respect to research support provided by the National Institutes of Health, Grant Number R01NS29225.

1.1 FIELD OF THE INVENTION

This invention relates generally to novel mammalian brain cell types and methods of culturing such cells. The methods of the instant invention, which utilize suspension cultures and factors that limit cell contacts, result in an amplification of the production of neural stem and progenitor cells, and clones of such cells, from the adult mammalian brain, including the human brain and from tissue with significant (*e.g.* 1 day up to at least 5 days) postmortem intervals. Propagation of neural stem and progenitor cells is relevant to the large-scale production of glial and neuronal cells, and clones of such cells, as well as self-repair of the brain in neurological disease.

1.2 DESCRIPTION OF THE RELATED ART

Prior to the present invention, cells from numerous tissues have been described that have attributes of stem of germ cells (*i.e.* spermatozoon or an ovum), and that are extremely well suited for rapid self-renewal. Brain-derived stem cells have only recently been a major focus of attention, using a variety of lineage tracing and culture methodologies. See, Gage *et al.*, 1995; Svendsen *et al.*, 1995, Alvarez-Buylla *et al.*, 1995, Steindler *et al.*, 1996 and Brustle *et al.*, 1995.

Previous studies showed the presence of a dense extracellular matrix ("ECM") on and around subependymal zone ("SEZ") cells of the adult rodent (see, Gates *et al.*, 1995 and Thomas *et al.*, 1996). ECM molecules may facilitate cell movement and aspects of differentiation during development, and they are also implicated in a number of neuropathological conditions. Glycoproteins such as tenascin-C (TN) and proteoglycans such as the chondroitin sulfate-containing proteoglycans (CSPG) are expressed in high levels in the young brain, where they seem to have a role in forming glycoconjugate-rich boundaries around different functional groups of neurons, such as the somatosensory whisker barrel fields and striosomes in the striatum. They are then down-regulated in later stages of development (*e.g.* postnatal days 14-21) and normal adulthood, but their expression is enhanced in neuropathologic conditions, such as traumatic brain injury, where they are an important component of glial scar formation. In the astroglial/mesonchymal scar, they may create a barrier that inhibits the growth of neurites into the scar, although it has been proposed that some ECM molecules may actually encourage neuritic growth under some circumstances. It has also been suggested that ECM molecules regulate cell proliferation, differentiation, migration, and survival through cell-cell and cell-ECM interactions.

Stem cells have been described in embryonic and postnatal mouse brain and in proliferative "neurospheres" that can be harvested and cultured from different brain areas, including the developing subventricular zone. See, Cattaneo *et al.*, 1990; Richards *et al.*, 1992; Reynolds *et al.*, 1992; Reynolds *et al.*, 1992; Reynolds *et al.*, 1996; Vescovi *et al.*, 1993; Kirschenbaum *et al.*, 1994; Kirschenbaum *et al.*, 1995; Fillmore *et al.*, 1996; and Gritti *et al.*, 1996.

Evidence from immunolabeling and cell birthday analyses has pointed to the existence of such cells in the adult SEZ. See, Luskin *et al.*, 1993; Menezes *et al.*, 1994; Levison *et al.*, 1993; Gates *et al.* 1995; Zerlin *et al.*, 1995; Thomas *et al.*, 1996; and Jankovski *et al.*, 1996. The combination of stem/precursor cells, and a dense ECM in the peri-ventricular SEZ throughout the neuraxis has prompted the inventors of the instant invention to refer to this area as being the neuropoietic "Brain Marrow" (Steindler *et al.*, 1996) since it contains elements in common with hematopoietic bone marrow.

In addition, it has recently been described that small numbers of neurons were found to arise from precursor cells of adult human temporal lobe (Kirschenbaum *et al.*, 1994; Laywell *et al.*, 1997). The production of proliferating progenitor cells from the adult rodent brain and spinal cord has also been recently described (see, Gritti *et al.*, 1996; and Weiss *et al.*, 1996). This is surprising in that with few exceptions, neuronogenesis has traditionally been thought to end shortly after birth in the mammalian central nervous system (CNS) (see, Gage *et al.*, 1995). The possibility that multipotential stem cells persist in the adult brain has implications for neuroregeneration and CNS transplantation. Accordingly, there is a need in the art for such technology. This need is met by the present invention.

2.0 SUMMARY OF THE INVENTION

The present invention discloses an advancement in the biological arts in which previously unknown brain stem cells are cultured and isolated. The multipotent brain stem cells are characterized in that the daughter cells of the brain stem cells differentiate into neurons and glia and, therefore, are useful in neuroregeneration cell biology, and CNS drug-effects and drug-discovery studies. A novel method of using such cells comprises culturing dissociated adult mammalian brain or postnatal tissue obtained from humans upt to two years of age in conditions that affect cell-substrate and cell-cell contacts. The cultured aggregates survive transplantation to the adult mammalian brain. Following transplantation, the daughter cells of the transplanted stem cells differentiate into other cell types, including but not limited to glia, such as astrocytes and oligodendrocytes and neurons, thus allowing for replacement of cells damaged by injury or disease.

Cells have been isolated from the postnatal and adult brain that can grow as proliferative clones, called neurospheres (Reynolds and Weiss, 1992), that are multipotent, giving rise to neurons, astrocytes, and oligodendrocytes. These cells can be described as neural stem cells (NSC's). A persistent population of neural stem cells (NSC's) in the postnatal and adult CNS is important not only as a model of basic neurodevelopmental processes, but also as a potential source of transplantable cells for the treatment of neurological injury and neurodegenerative disease. A crucial step in

understanding the biology of NSC's is their *in vivo* localization and identification. It has been shown that the periventricular subependymal zone (SEZ) is the site of frequent neurogenesis, and this region presumably contains the highest concentration of NSC's in the postnatal and adult brain (Weiss *et al.*, 1996). Ciliated ependymal (CE) cells, which line the ventricular system immediately subjacent to the SEZ are one possible source of NSC's. The suspicion that CE cells might be capable of generating new neurons in adult mammals (Altman, 1962) is supported by studies showing the production of neurons by ependymal cells in models of CNS injury in lower vertebrates (Anderson and Waxman, 1985; Molowny *et al.*, 1995).

Recent studies addressing the role of CE cells in mammalian neurogenesis have reached conflicting conclusions. It has been reported (Johansson *et al.*, 1999) that individual CE cells of rodents are NSC's since they form neurospheres when cultured in isolation, and since some CE cells appear to divide in response to injury. However, a later study (Chiasson *et al.*, 1999), found that CE cells, while capable of forming neurosphere-like clones *in vitro*, are unipotent since they give rise only to astrocyte-like cells. Yet a third study (Doetsch *et al.*, 1999) failed to show the formation of CE cell-derived clonal structures at all. Adding to the complexity, the latter study seemed to indicate that a special type of astrocyte residing within the SEZ may have NSC attributes.

In the present work, studies of the ECM molecules in the adult brain revealed the existence of an ECM-rich pathway within which neuronal progenitor cells proliferate and migrate. These ECM molecules play a significant role in these events. According to the invention, the *in vitro* manipulation of these and related molecules affects cellular adhesity to other cells or substrates, and affects the growth of neural stem and progenitor cells, as described below.

To discourage cell-cell interactions that induce cellular differentiation, and thus contribute to an increased cellular proliferation of sternlprogenitor cells, dissociated cells ftom the adult brain were cultured in factors that interfere with protein-protein interactions, or in gelatinous organic substances (*e.g.* methylcellulose) that also discourage cell contacts and allow the isolation of clonally-related colonies (spheres) of cells. There is a surprisingly large expansion of the numbers of adult brain stem and progenitor cells due to these conditions, with potentially up to millions of neuronal and

glial progenitors from small numbers of founder cells in less than three months. When aggregates of progenitor cells are plated on particular extracellular matrix molecule substrates in the presence of different growth factors, hormones, steroids, and other factors (see Examples 3 and 10), they differentiate into neurons and glial cells. Such cells are suitable for studies of drug-discovery and testing using clones of glial and neuronal cells as well as for cell replacement therapies in a variety of brain structures *e.g.*, in the brain or spinal cord for regeneration or space-occupation, as in spinal cord syrinx injuries, or stroke cavities, arteriovenous malformations, epileptic foci, or peripheral nerve neuromas.

Stem cells appear to make up 0.001-0.01% of an entire population of cells in renewing or potentially renewing tissues such as bone and "brain marrow" (Thomas *et al.*, 1996). Hence, any method that assures a large scale production of differentiated cells from a small number of the most primitive stem/precursor cells is extremely useful for self-repair (autologous cell replacement) therapies following traumatic or degenerative disease. The invention harvests brain cells from a variety of sources including, but not limited to, mammalian brain specimens, human brain biopsy, and postmortem mammalian brain tissue. The invention method includes isolation and amplification of neural cells as a therapeutic self-repair approach for neurological disease, particularly human disease. To accomplish the isolation and amplification, the present invention uses a novel tissue culture approach to maximize the isolation of stem cells, and to assure a maximal number of transplantable cells from a very small number of stem cells, thus assuring the least risk for complications from a targeted and small biopsy site. At the same time, the culture method offers the possibility for large-scale cell production from a very limited number of extracted stem cells.

Furthermore, since a more primitive type of stem/precursor cell, than any seen prior to now, has been isolated and expanded using the invention technology, this cell type is more amenable to central nervous system (CNS) transplantation and integration, as well as gene transduction approaches. The new cell type exhibits clonogenic properties and has the attributes of a multipotent astrocyte stem/progenitor cell.

In certain embodiments, for example, using various vectors, the isolated cells of this invention can be transduced with genes coding for desired functions. For example,

vectors containing genes coding for adhesion molecules can be transduced into these cells to increase cellular adhesivity. This would be useful, for example, to produce glia with increased adhesivity for cavity-filling approaches. Alternatively, the isolated cells of the present invention can be transduced with genes coding for growth factors and directing neuronal phenotype, including neurotransmitter associated genes to produce neurons capable of increased neurotrasmitter production. In addition, because the cells of the present invention are multipotent, they are extremely well suited for cell-grafting approaches.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory and are intended to provide further explanation of the invention as claimed. Other objects, advantages and novel features will be readily apparent to those skilled in the art from the following detailed description of the invention, when considered in conjunction with the accompanying drawings.

The accompanying drawings, which are included to provide a further understanding of the invention, are incorporated in and constitute a part of this specification. They illustrate several embodiments of the invention and, together with the written description, serve to explain the principles of the invention.

3.0 BRIEF DESCRIPTION OF THE DRAWINGS

The drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1A, FIG1B, FIG. 1C, FIG. 1D, FIG. 1E and **FIG. 1F** show phase contrast and electron microscopic images of type I, II, and III clones. **FIG. 1A, FIG. 1C**, and FIG. 1E are phase contrast images of type I, II and III clones of cultured adult brain cells, respectively. **FIG. 1B, FIG. 1D,** and **FIG. 1F** show type I, II and III spheres counterstained with propidium iodide, respectively. Scale bars for **FIG. 1A, FIG. 1B, FIG. 1C, FIG. 1D, FIG. 1E** and **FIG. 1F** are 40, 3 0, 20, 30, 20, and 30 microns, respectively.

FIG. 2 depicts the types of spheres found in the culture paradigm of the invention, and the generation conditions for the appearance and evolution of sphere types from brain.

FIG. 3A, FIG. 3B, FIG. 3C and **FIG. 3D** show phase and electron microscopic images of type II (**FIG. 3A** and **FIG. 3B**) and type III (**FIG. 3C** and **FIG. 3D**) spheres. Scale bars for **FIG. 3A, FIG. 3B, FIG. 3C** and **FIG. 3D** are 10, 5, 15, and 2 microns, respectively.

FIG. 4A, FIG. 4B, FIG. 4C, FIG. 4D, FIG. 4E, FIG. 4F, FIG. 4G, FIG. 4H, FIG. 4I and **FIG. 4J** show immunostaining of early and late type II and type III spheres. Scale bars for **FIG. 4A, FIG. 4G** and **FIG. 4J** are 10 microns, **FIG. 4B** and **FIG. 4C** are 15 microns, **FIG. 4E** and **FIG. 4F** are 30 microns, **FIG. 4H** is 20 microns, and **FIG. 4I** is 100 microns.

FIG. 5A, FIG. 5B, FIG. 5C and **FIG. 5D** show the evolution and proliferation of type II (**FIG. 5A** and **FIG. 5C**), and type III (**FIG. 5B** and **FIG. 5D**) spheres. Scale bars for **FIG. 5A** and **FIG. 5B** are 25 microns, and for **FIG. 5C** and **FIG. 5D** are 10 microns,

FIG. 6A and **FIG. 6B** show type II and type III spheres from ROSA-26 transgenic mice. Scale bars for **FIG. 6A** is 50 microns, and **FIG. 6B** is 30 microns.

FIG. 7A, FIG. 7B, FIG. 7C and **FIG. 7D** show phase and electron microscopy of a type II adult mouse and type II adult human sphere. The adult mouse sphere is approximately 100 microns in diameter, while the adult human sphere is approximately 200 microns in diameter.

FIG. 8A, FIG. 8B and **FIG. 8C** show astrocyte monolayers from postnatal day 2 mouse cerebral cortex generate spherical clones. **FIG. 8A** shows phase micrograph of a confluent layer of astrocytes. Scale bar = 50 μm. Early passage monolayers often

contain a small number of microglia, as seen in the lower right inset using immunofluorescence for Mac-1 (FITC), counterstained with propidium iodide (PI). Scale bar in lower right inset = 10μm. Astrocyte monolayers from this age also generate spherical clones when grown in suspension culture with EGF and bFGF (upper left inset, Scale bar = 100μm; and inset in **FIG. 8B**, showing a scanning electron micrograph of such a clone after plating on a laminin-coated substrate; Scale bar 50μm). Similar spherical clones are generated when single astrocytes are cultured in isolation (phase micrograph of clone derived from a single astrocyte is shown in the upper right inset, Scale bar = 30μm). **FIG. 8B** shows GFAP immunolabeling and PI counterstaining of most but not all astrocytes within the monolayer. Scale bar = 25μm. **FIG. 8C** shows Spherical clones are generated from monolayers such as these in which virtually all cells within the monolayer exhibit immunolabeling for the immature astrocyte marker, vimentin (PI counterstaining) and S100β (inset). Scale bar = 25μm in **FIG. 8C** and 10μm in the inset.

FIG. 9A, FIG. 9B, FIG. 9C, FIG. 9D, and **FIG. 9E** shows astrocyte-derived neurospheres are proliferative and express neuronal and glial antigens. **FIG. 9A** shows BrdU and GFAP immunofluorescent double-labeling of a sphere derived from a P2 cortical astrocyte monolayer, showing the highly proliferative nature of these sphere cells. BrdU was present in the culture medium for 24 hours prior to plating on a coverslip. Scale bar = 50μm. Upper left inset: Low magnification BrdU immunofluorescence labeling of an entire, attached, differentiating neurosphere. Scale bar = 50 μm. Lower right inset shows double immunolabeling for A2B5 (oligodendrocyte precursor marker) and GFAP, PI counterstained, of differentiating cells from an astrocyte-derived neurosphere. Scale bar = 10 μ. **FIG. 9B** shows immunolabeling of a single P7 cerebellar astrocyte-derived neurosphere showing MAP-2 positive cells, indicating the presence of neurons within these clones. The inset in **FIG. 9B** shows a higher magnification of a single MAP-2 positive immature neuron within the neurosphere. Scale bars = 30μm in **FIG. 9B**, and 10μm in the inset. **FIG. 9C** shows GFAP and β-III tubulin, PI counterstained, double immunolabeling of a neurosphere derived from a P1 cerebral cortex astrocyte monolayer. Note the beta-III tubulin

immunopositive neurites arborizing around PI-stained cells, and GFAP astrocytic processes within the lower portion of the neurosphere. Scale bar – 10μm. **FIG. 9D** shows β-III tubulin immunolabeling of a neurosphere derived from a P1 spinal cord astrocyte monolayer. Note two β-III tubulin positive immature neurons at the edge of the neurosphere, compared to apparently more mature immunolabeled neurons (upper right and lower left insets) that have migrated away from the neurosphere and elaborated long processes. Scale bars = 10μm in **FIG. 9D** and both insets. **FIG. 9E** shows L1 neuronal immunolabeling (and PI counterstaining) of a neurosphere from a P1 spinal cord astrocyte monolayer, showing pervasive labeling of presumed immature neurons throughout the neurosphere. Scale bar = 25μm.

FIG. 10A, FIG. 10B, FIG. 10C, FIG. 10D, FIG. 10E, FIG. 10F, and **FIG. 10G** show alkaline phosphatase (AP) enzyme histochemistry and immunocytochemistry of cells and neurospheres derived from *Gtv-a* transgenic mouse astrocytes infected with RCAS-alkaline phosphatase, showing cells expressing both AP and neuronal phenotype markers. **FIG. 10A** shows a P2 astrocyte monolayer after infection with the avian leukosisvirus expressing the AP reporter gene, showing infected astrocytes with varying morphologies (*e.g.* arrow). Upper right inset shows AP histochemistry of the DF1 chick embryo fibroblast line engineered to produce the RCAS-AP leukosisis virus. Lower right inset shows a single infected astrocyte, double exposure, with both AP histochemical labeling (black punctae) and GFAP immunofluorescence (FITC). **FIG. 9B** shows a neurosphere derived from a *Gtv-a* astrocyte monolayer. AP histochemistry reveals cells of this neurosphere expressing the RCAS-AP gene, thus indicating their derivation from a single, infected astrocyte. Upper right inset shows an example of a neuron derived from such a neurosphere, immunofluorescence for β-III tubulin (FITC). Lower pair of insets show the same neurosphere expressing AP (left) and MAP-2 (right), revealing numerous MAP-2 positive processes emanating from an RCAS-infected neurosphere. Scale bars = 100μm in **FIG. 10B**, 50μm in the lower insets, and 40μm in the upper inset. **FIG. 10C** and **FIG. 10D** show a single RCAS-AP infected neuron that has migrated and differentiated away from its neurosphere. Brightfield labeling of AP reaction product (dots) in this neuron (**FIG. 10C**), co-localized with β-III tubulin immunofluorescence

(FITC) shown in **FIG. 10D**. Asterisk marks the nucleus of this cell in each figure. Scale bars in **FIG. 10C** and **FIG. 10D** = 10μm. **FIG. 10E** shows low (**FIG. 10E**) and high (lower inset) magnification of a RCAS-AP infected neurosphere, double exposure, showing β-III tubulin positive neurons (FITC) within a densely AP positive neurosphere. Arrow points to double labeled neurons within the neurosphere, exhibiting both labels. Asterisks mark the top edge of the neurosphere. Scale bar = 10μm, **FIG. 10F** and **FIG. 10G**. A double labeled neuron at the edge of a neurosphere (the neurosphere is in the upper left portion of the field), as seen in single exposures of the same field, brightfield for AP in **FIG. 10F** and immunofluorescence for β III-tubulin in **FIG. 10G**. The asterisks are within the nucleus of this double labeled cell in both images, and other nuclei appear as sparsely-positive AP ovals. Scale bars = 10μm.

FIG. 11 is a summary of the neurosphere-generating potential of astrocyte monolayers derived from different CNS regions, from animals of different ages. Note an apparent critical period at P10/11 when astrocytes derived from the cerebral cortex, cerebellum and spinal cord cease to generate neurospheres, while astrocytes from the subependymal zone do not observe this critical period and retain the ability to generate neurospheres well into adulthood.

FIG. 12A, FIG. 12B and **FIG. 12C** show fibroblast monolayers, derived from mouse muscle, form spherical clones when replated in suspension culture supplemented with EGF and FGF2 (arrowheads in **FIG. 12A**). When attached and allowed to differentiate, fibroblast-derived clones give rise only to cells with fibroblast-like morphology (**FIG. 12B**), and these cells are never immunopositive for neural-specific antigens. Monolayers of microglia (**FIG. 12C**) fail to generate spherical clones when replated in suspension culture supplemented with EGF and FGF2.

FIG. 13A, FIG. 13B, FIG. 13C, FIG. 13D, FIG. 13E, FIG. 13F and **FIG. 13G** show brain tumor-derived clones. When stem/progenitor cells are harvested and cultured from an adult human brain anaplastic astrocytoma (this example, but also from astrocytomas and glioblastomas) both astrocytes (immunofluorescence for astrocytic glial

fibrillary acidic, GFAP, protein) and neurons (immunofluorescence for neuronal β-III tubulin) arise from multipotent neurospheres in suspension culture (with semi-solid, *i.e.* methylcellulose-containing media) and when plated on laminin or poly-L-ornithine. **FIG. 13A** are two attached clones from this anaplastic astrocytoma seem to merge, with neurons (bright cells) and astrocytes (darker stained cells) within and surrounding the clones. **FIG. 13 B** are two clones immunostained for just neuronal β-III tubulin and shows many neurons (brightly immunofluorescent cells) that arise from such glial tumors. **FIG. 13C** are two different clones, from the same tumor, again stained for the same astrocytic (dark) and neuronal (bright cells) markers. **FIG. 13D** is a single, beta III tubulin positive neuron that is outside of the attached clone, and exhibits unusual somatodendritic morphology. **FIG. 13E** shows higher magnification of neurons and astrocytes with apparently normal morphologies. **FIG. 13F** and **FIG. 13G** show the same field of cells immunostained for GFAP (**FIG. 13F**) and neuronal β-III tubulin (**FIG. 13G**). It can be seen that there are single cells expressing both neuronal and glial antigens, which is not normally seen within clones derived from non-tumor stem/progenitor cells.

4.0 DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present invention describes methods which can be used to isolate, amplify, and grow stem/precursor cells from the mammalian brain. Some of these stem/progenitor cell are multipotent astrocytes or tumor cells. By manipulating specific aspects of cell separation and cell adhesion, the invention methods described herein can be used to specifically isolate and culture type I, type II and type III precursor cells. It should be noted that in a recent paper by Kukekov *et al.* (*alia*, 1997 the type II and type III cells of the instant invention are referred to as type I and type II cells, respectively, for convenience. Types II and III appear to be more mature stages of type I cells.

It should also be noted that type I, II or III stem cells of the instant invention are the cells which generate type I, II, and III clones/aggregates/neurospheres, respectively. The term clone or aggregate will be used to refer to these cell aggregates generated from a single cell.

Cell separation and cell adhesion can be manipulated using a variety of contact-limiting and contact-inhibiting factors. For example, chemical-separating agents such as mercaptoethanol, physical separating agents such as methylcellulose, and anti-adhesives such as poly 2-hydroxyethyl methacrylate are used to deter cell-cell and cell-substrate associates during the initial isolation of stem/precursor cells from the newly-dissociated brain. This allows the "purification" of these cells from mature, differentiated neurons and glia that are also dissociated during the brain dissociation procedures. The mature, differentiated neurons and glia cannot survive these anti-adhesion, anti-cell interaction procedures. Thus, agents such as mercaptoethan are always used in the first stage of isolation of type I and II clones to help deter the survival of the more mature cellular elements by deterring their clustering. At the same time, agents such as mercaptoethanol may have certain growth-promoting actions on the single stem/precursor cells that eventually proliferate to form these early sphere types.

Since cell-cell and cell-substrate interactions are important for cellular differentiation, contact-inhibiting (or contact-limiting) factors as mercaptoethanol are eventually removed from the culture medium for the evolution or differentiation of type II and type III spheres. The differentiation of type III spheres requires other additional factors, including growth factors such as beta fibroblast growth factor, epidermal growth factor, or similar factors that are also contained within pituitary extract. Such additional factors are described in the type III culture media discussed below, see Example 3.

The adult mammalian brain harbors a discrete, prolific population of primitive stem/precursor cells that possess many of the attributes of stem/precursor cells seen in other organs. Since these earliest (most primitive) cells do not readily attach to culture substrata, and require contact-limiting factors for amplification, they may have been overlooked in previous culture studies of adult brain stem and progenitor cells. Under certain culture conditions, these cells evolve into many different classes of cells, including progenitors that give rise to neurons and different types of glia. In this way, they possess many of the cytological characteristics of hematopoietic stem/precursor cells that can give rise to different types of blood progenitor cells. These brain stem/progenitor cells may be produced on a large scale and can be genetically altered using a variety of transfection methods.

The multipotential brain cells of the instant invention can be directed to particular neuronal or glial lineages. Thus, the culturing methods of the instant invention can be used to produce large numbers of clonally related cells of a specific cell type. The methods of producing such large populations of cells as well as the cells themselves are specifically useful in replacing cells that are lost due to disease processes. For example, the production of large quantities of specific glial cells by the methods of the present invention and subsequent transplantation of these glia into the brains of multiple sclerosis patients, would be particularly beneficial to such patients. Similarly, neurons generated from the stenvprecursor cells of the instant invention, transplantation of these neurons, and the integration of the activities of the replaced cells into established brain circuitries is particularly useu in brains where neuronal cell loss has occurred. Cells generated using the methods of the instant invention can also be used in the brain or spinal cord for regeneration or space-occupation, as in spinal cord syrinx injuries, or stroke cavities, arteriovenous malformations, epileptic foci, or peripheral nerve neuromas to fill cavities. Large or small scale production of the cells using the invention methods are also useful for drug-discovery and testing.

The culture paradigms described below yield three morphologically and antigenically distinct populations of cellular aggregates, types I, II and III. These populations of cellular aggregates are termed "clones", each clone having distinct characteristics (FIG. 1). As shown in FIG. 1A, clones of type I appear as phase-bright, very small dense bodies. It is not possible to discern individual cells within the clones using phase microscopy. However, when the cells are counterstained with propidium iodide (PI) (FIG. 1B), type I clones exhibit areas of very small, punctate staining interspersed with regions that lack staining. Type I clones do not attach to either plastic or laminin-coated substrates, and cells of individual clones are not separable by trypsinization. Furthermore, type I clones are immunonegative for all of the cell-specific markers tested so far.

Type II clones from adult mouse and human brain dissociations, spontaneously appear in suspension cultures (FIG. 1C, FIG. 1D, FIG. 3A, and FIG. 3B), containing at least one contact-limiting or contact-inhibiting factor. Some exemplary contact limiting factors include, but are not limited to, mercaptoethanol, poly 2-hydroxyethyl

methacrylate, and methylcellulose. Mercaptoethanol functions as a chemical-separating agent as it breaks disuffide linkages of proteins which are involved in cell-cell and cell-substrate interactions. Methylcellulose functions as a physical separator; it is a viscous bioorganic solution which, by its viscous nature, limits contacts between cells and between cells and a substrate, and allows clonal analyses. Poly-2-hydroxyethyl methacrylate functions as an anti-adhesive for substrate coating. This prevents cell contact with adhesive surfaces and also prevents differentiation. Other compounds or procedures which function to limit or inhibit cell-cell and cell-substrate interactions can also be used with this invention. Culturing as a suspension also deters cell-cell and cell-substrate contacts with higher yields of spheres, but culturing in methylcellulose again, allows clonal analyses.

In contrast to type I clones, type II clones are phase-dark, spherical bodies that become larger with time (FIG. 1C and FIG. 3A). Electron Microscopy (EM) revealed that type II clones consist of rings of small, tightly apposed cells that surround a core of flocculent, non-cellular material having many characteristics of extracellular matrix (FIG. 1D and FIG. 3B). The type II cell has many organelles, including endoplasmic reticulum, Golgi apparatus, dense bodies, and mitochondria. The closed arrow in FIG. 1C points to a multisphere aggregate as shown by propidium iodide counterstaining, a DNA stain for cell nuclei, (FIG. 1D). The closed arrow in FIG. 1E points to a type II sphere residing near a type III sphere, which is marked by an open arrow.

Type II clones do not attach to either plastic or laminin-coated substrates. Immediately after they appear in culture, type II clones are immunonegative for cell-specific markers, including GFAP (glial fibrillary acidic protein, which labels more mature and reactive astrocytes), nestin (RAT-401, which stains neurepithelial stem cells as well as radial glia), and TuJI (which stains class III β-tubulin that is expressed in recently postmitotic (committed) as well as mature neurons in the CNS). However, after approximately ten days to two weeks *in vitro*, some cells of type II clones (late type II, or early type III) become immunopositive for nestin, but remain immunonegative for GFAP and TuJ1 (FIG. 4). If the factors that inhibit cell contact are removed from the medium, provided the type II clones are not kept in the contact-limiting factor for more than two weeks, type II clones will convert to type III clones.

In contrast to type II clones, type III clones, when plated on plastic or laminin-coated substrates, attach readily, and produce a number of process-bearing cells that migrate away from the sphere to form a single layer of cells. Type III cells are immunopositive for a variety of cell-specific markers, including GFAP, nestin, L1 (a marker of an adhesion molecules that is on the surface of neurons and their processes), and TuJ1 (FIG. 4). Some cells exhibit very light, punctate staining using an antibody to the 04 antigen (marker of oligodendrocytes).

In addition to the type I, type II and type III clones, the primary cultures of the instant invention also appear to initially contain differentiated neurons and glia. However, in the presence of contact-inhibiting factors, neurons and astrocytes were not immunologically detected after 1 week; they presumably cannot survive under these culture conditions.

The culture paradigm of the invention, and the generation conditions for the appearance and evolution of sphere types from a brain are depicted in FIG. 2. Both type II and type III stem/precursor cells are generated from a dissociated adult mammalian brain. Type II cells appear 2-3 weeks after mercaptoethanol, or other cell contact limiting factor, is added to the culture medium. When mercaptoethanol, or other contact-limiting factor, is subsequently removed from the medium after the appearance of type II spheres, the type II spheres increase in size and, after an additional 10-14 days, convert to type III spheres. In addition to developing from type II clones after the addition and subsequent removal of a contact-limiting factor, type III clones can also appear spontaneously in suspension cultures grown in the absence of contact limiting factors.

FIG. 3 shows the phase and electron microscopic images of type 11 (FIG. 3A and FIG. 3B) and III (FIG. 3C and FIG. 3D) spheres. After approximately 30 days *in vitro*, type II clones take on characteristics of type III clones. The size of the type II clones increases dramatically, and their color changes from phase-dark (FIG. 1C and FIG. 3A) to phase-bright (FIG. 1E and FIG. 3C). In addition, large cells appear at the edges of the type III clones.

FIG. 3C shows type III spheres which are generally larger and phase-brighter than type II spheres. The open arrow points to a late type II or early type III sphere, and the closed arrow points to a large cellular protrusion on the periphery of a late type III sphere

which appears brighter than the early type III, and has a more irregular border due to many protrusions. FIG. 3D shows cells of type III spheres which look more mature than type II cells. The open arrow points to a large cell, and the closed arrow marks a smaller cell with a darker nucleus.

Type III spheres will continue to increase in size and begin to display cellular heterogeneity when grown in suspension culture. Eventually, cells within suspended type III spheres begin to express markers of differentiation. Such cells can eventually become neurons and glia when plated onto favorable substrata such as laminin/poly ornithine coated surfaces, polylysine, or plastic, or other matrix molecules, or transplanted into a host brain.

FIG. 4 shows the immunostaining of early and late type II and III spheres illustrating that the various characteristics of the type II and type III spheres can be readily distinguished from their immunostaining profiles and their phase-contrast, and EM images. Early type II spheres are shown in FIG. 4A. The open arrows point to two type II spheres that abut each other. These spheres are negative for the putative stem cell intermediate filament protein, nestin. A more mature type II sphere that is immunopositive for nestin is shown in FIG. 4E. FIG. 4C shows an early type III sphere immunopositive for nestin, with some unlabeled cells also apparent. FIG. 4D shows a phase contrast image of a late type III sphere (open arrow) showing processes after attaching to plastic. FIG. 4E and FIG. 4F show a type III sphere, with cells beginning to disperse after attaching to plastic. This sphere is double immunostained for GFAP (FIG. 4E) and O-IH tubulin (FIG. 4F). FIG. 4G depicts astrocytes and shows that dispersed cells of type III spheres are immunopositive for nestin. FIG. 4H shows astrocytes from dispersed type III spheres also stain for GFAP. The open arrow points to a morphologically different astrocyte than the large cell on the right. FIG. 4I shows cells that are dispersed from an attached type III sphere and which are immunopositive for L1. The arrow points to a long L1-positive process. FIG. 4J shows a single cell immunopositive for β-tubulin, counterstained with propidium iodide, after attachment of a type III sphere. Thus, type II and type III spheres are easily distinguishable from each other, not only by the differences in phase contrast, and EM images, but also by their distinct immunostaining profiles.

The evolution and proliferation of a type II sphere differentiating into a type III sphere can be seen in FIG. 5. A single late type II sphere (arrow in FIG. 5A) was followed in suspension culture with phase microscopy. After 10 days, this type II sphere had increased in size and altered its morphology to become the type III sphere shown in FIG. 5B. The open arrowhead points to a cellular protrusion on the edge of the sphere typical of the type III sphere. Cellular debris around the type II sphere in FIG. 5A is absent from the type III sphere in FIG. 5B due to movement during feeding. Heterogeneous cultures of spheres exposed to a 20 hour pulse of BrdU reveal proliferative cells in both type II (FIG. 5C) and type III (FIG. 5D) spheres. Both the small sphere in FIG. 5C and the larger sphere in FIG. 5D contain labeled nuclei with a variety of sizes. This indicates that there may be different progenitor cells types proliferating in the spheres; some progenitor cell types giving rise to neurons, some giving rise to glia.

Removal of the contact inhibiting factors allows differentiation to occur. In addition, the type II and type III growth media containing growth factors such as basic fibroblast growth factor (bFGF), or epidermal growth factor (EGF) encourages differentiation.

Other growth factors such as brain-derived neurotrophic factor (BDNF), glial derived neurotrphic factor (GDNF), NT3, and ciliary neurotrophic factor (CNTF) may also encourage differentiation of the stem/precursor cells.

It should also be noted that the flat, spread appearance of the type III sphere in FIG. 5D is probably due to this sample being taken from a 96 hour culture as opposed to the 48 hours for the type II sphere, and type III spheres have a tendency to spread and migrate when attached. The lack of spreading of the type II sphere may also account for the higher level of background staining.

Type II and type III clones were also generated from ROSA-26 mice, a strain that expresses the β-galactosidase transgene in all cell types, to perform transplant experiments. ROSA-26 transgenic mice are commercially available from the Jackson Laboratories, Bar Harbor, Maine. The results show that following transplantation into adult mouse brain, type II and type III clones can survive and differentiate. (FIG. 6). When these β-galactosidase positive type II and type III clones were transplanted to the

strialum of adult ICR mice, small and large X-gal positive cells were found to survive up to two weeks. Moreover, immunocytochemistry with GFAP revealed the presence of labeled astrocytes and non-labeled larger cells (presumably neurons). FIG. 6A shows a β-galactosidase positive late type III sphere (large open arrow), and unlabeled early (right open arrowhead) and late (left open arrowhead) type II spheres. The filled arrow points to unidentified-labeled elements out of the plane of focus. FIG. 6B shows the combined bright field/fluorescence image of a transplant of type II and III spheres into the striatum of an adult ICR mouse. Surviving astrocytes marked with a filled arrow are counterstained for GFAP. Large GFAP-negative, β-galactosidase- positive cells, presumed to be neurons (open arrow), are also seen. Other transplanted cells can be seen within this host brain structure as well. Immunofluorescence is light in color; the β-galactosidase product is dark in color. These results support the conclusion that the novel stem/precursor cells are useful in regeneration following neurological cell damage.

Type II and type III clones were also generated from the adult human brain, and from dead animals with long postmortem intervals when the animals were kept at 4°C (FIG. 7). FIG. 7 shows phase and electron microscopic images of type II adult mouse and human spheres. FIG. 7A shows the phase microscopic image of a type II adult mouse sphere that looks similar to a sphere with a postmortem interval of 0 hours, while FIG. 7B shows the phase microscopy of a type II sphere from an adult mouse with a postmortem interval of sixteen hours. FIG. 7C shows the electron microscopic image of a type II sphere from an adult transgenic mouse (tenascin glycoprotein knockout mouse). A complex cellular aggregate can be seen. This sphere is approximately 100 microns in diameter. FIG. 7D shows the electron microscopy of an adult human sphere (as shown in the inset, a phase microscopic image of a type II sphere). Similar to the electron microscopy of a type II sphere from an adult transgenic mouse (FIG. 7C), this human sphere (FIG. 7D) also shows a complex cellular aggregate. This human sphere is approximately 200 microns in diameter.

The different types of clones observed in the cultures described above and in the experiments described below, represent a continuum of cell proliferation and differentiation, with the existence of both early and late type II clones, which can be compact or loose in appearance, based on cell packing density, that eventually

differentiate into type III clusters (FIG. 1 and FIG. 3). The potential for numerous, undefined hematopoietic stem cells still exists (see, for example, Larochelle *et al.*, 1996). The identification and understanding of neuropoietic stem and progenitor cells based on a combination of morphology, gene marking, and unique biochemical features, such as that described herein, ensure reliability in the results. The use of just one feature as an identification tool can occur, although it makes the recognition of the specific stem cell type rather tenuous.

While the inventors do not wish to be bound by any particular theory, neuropoiesis in the adult brain is probably a rather limited event, based on current knowledge of hematopoiesis and the presence of stem cells in other tissues, as well as the apparent existence of a quiescent population of so-called stem cells in the SEZ (see, Potten *et al.*, 1990 and Morshead *et al.*, 1994). For this reason, methods that amplify the *ex vivo* production of these cells, as described herein, are useu to generate large numbers of such cells for classification and transplantation. Furthermore, the methods of the instant invention can be applied to harvesting the small number of stem/precursor cells from adult brains, particularly adult brains with significantly long postmortem intervals (*e.g.* 1-5 days), allowing the "banking" of these cells for future studies and cell-replacement therapies.

Also, novel approaches as described here, that uncover either novel stem/precursor cells or aspects of their growth and differentiation that lead to classification of stages of adult brain neuropoiesis are also useful. The described methods allow this process with the production of the most primitive stem/precursor cells, and facilitate the generation and analyses of a continuum of developing and differentiating brain stem/precursor cells.

The culture conditions of the present invention involve limiting cell-cell and cell-substrate interactions leading to the enhanced production of spheres of type II and III cells. Since previous studies have only shown a dense extracellular matrix within the SEZ *in vivo* (see, for example, Gates *et al.*, 1995 and Thomas *et al.*, 1996) and since ECM molecules have been reported to affect the proliferation and differentiation of hematopoietic stem cells (see, for example, Klein *et al.*, and Yoder *et al.*, 1995) and while the inventors do not wish to be bound by any particular theory, it is presumed, that the

methods of the present invention affect the actions of the ECM molecules, adhesion proteins, growth factors and their interactions. Preliminary studies using immunocytochemistry and the reverse transcriptase polymerase chain reaction (rtPCR) suggest that sphere cells express transcripts as well as protein of particular ECM molecules (*e.g.* tenascin).

As described above, the appearance of two types of proliferating cells that form neurosphere-like aggregates from dissociated adult brain, termed type II, and III clones, were consistently observed. While the type III clones in the culture paradigm of the invention most likely represent neural progenitor cells that have previously been described in the aforementioned papers (see, for example, Reynolds *et al.*, 1992; Reynolds *et al.*, 1996; and Weiss *et al.*, 1996, all cited above, it has been heretofore unrecognized that such type III spheres are derived from the type II precursors of the instant invention. By isolating and amplifying type II spheres in specific culture conditions, type III spheres can be obtained. In addition, prior to the disclosure of the methods of the instant invention, type II and type III spheres have not been previously obtained from postmortem brain specimens.

In addition, prior art stem cells are not as primitive as the type II stem cells of the present invention as evidenced by the fact that the prior art stem cells are all nestin-positive. The type II clones of the instant invention represent a truly unique, ontogenetically earlier form of stem/progenitor than those previously described. This conclusion is supported by data showing that the type II clones are first initially nestin-negative, followed by a progression through a nestin-positive state to become a type III clone, and finally differentiation into neurons or glia (FIG. 2, FIG. 3 and FIG. 4).

The following examples are offered by way of illustration and are not intended to limit the scope of the claimed invention.

5.0 EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute

preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5

5.1 EXAMPLE 1 - THE PRODUCTION OF TYPE I CLONES

Adult ICR or transgenic or mutant mice, or biopsy specimens ftorn human temporal lobe (for epilepsy surgery), or brain specimens with significant (i.e. at least one day) postmortem intervals, were used as tissue sources for dissociations. The brains were 10 dissociated and cultured follows. Extracted brain tissues were minced with a razor blade and washed in a mixture of ice-cold DMEM (Dulbecco's modified Eagle's medium, commercially available from a variety of vendors) and any antibiotic-antimycotic product such as Sigma Chemical Co. Catalogue #A5955 (100×). (Such antibiotic-antimycotic products are also commercially available from Gibco Bri, Grand Island, NY). Brain 15 pieces were transferred to a beaker containing 0.25% trypsin and EDTA (ethylenediaminetetraacetic acid) and mixed on a magnetic stir-plate for 15 minutes, triturated with a plastic pipette, filtered through sterile gauze, and collected in a 15 ml tube and centrifuged for 5 minutes at 1200 rpm. Cells were resuspended in DMEM/F12 medium + N1 supplement (a standard tissue culture medium available from a variety of 20 vendors) plus 5% FBS (fetal bovine serum) and grown in suspension cultures by plating at high density on a non-adhesive substrate (tissue culture plastic coated with poly 2-hydroxyethyl methacrylate, Sigma Chemicals). Cells were fed every 3-4 days by centrifugation and resuspension in fresh medium.

The basic media for culturing type I cells comprises the following ingredients: 25 Insulin (5 μg/mL), putrescine (100 μM), progesterone (20 μM), sodium selenite (30 μM), pituitary extract (20 μg/mL), transferring (100 μg/mL), and 5% fetal calf serum (FCS) in DMEM/F12 media.

Type I cells only appear in suspension cultures containing a non-adhesive substrate such as poly 2-hydroxyethyl methacrylate. Some type II cells are also present 30 in these cultures.

5.2 EXAMPLE 2 - THE PRODUCTION OF TYPE II CLONES

Type II clones, similar to type I clones, were generated from adult ICR, transgenic or mutant mice, or biopsy specimens from human temporal lobe (for epilepsy surgery). In addition, Type II clones were also generated from the adult human brain, and from dead animals with long post mortem intervals when the animals were kept at 4°C.

The brains were dissociated and cultured as previously described for the generation of Type I clones. Briefly, extracted brain tissues were minced, washed, and transferred to a beaker containing 0.25% trypsin and EDTA. After being mixed on a magnetic stir-plate for 15 minutes, the culture was triturated with a plastic pipette, filtered through sterile gauze, centrifuged, and resuspended in DMEM/FI2 medium + NI supplement, plus 5% FBS, plus 20 μg/mL pituitary extract (from Gibco) and grown in suspension cultures by plating at high density on a non-adhesive substrate.

Cells were plated and fed as described above for the type I cells. However, the basic media described above (comprising insulin (5 μg/mL), putrescine (100 μM), progesterone (20 μM), sodium selenite (30 μM), pituitary extract (20 μg/mL), transferring (100 μg/mL), and 5% fetal calf serum (FCS) in DMEM/F12 media) also contained 10 ng/mL basic fibroblast growth factor (bFGF), and 10 ng/mL epidermal growth factor (EGF). Importantly, the culture media additionally contained 100 μM mercaptoethanol as a contact-limiting factor that reduces disulfide bonds (see, Herrington, 1986). Cultures contained dense debris for 10-14 days. Mercaptoethanol was then removed from the medium after 10-14 days. Clones of type II were present in these cultures. Some type III clones were also present.

5.3 EXAMPLE 3 - THE PRODUCTION OF TYPE III CLONES

Similar to the type I and type II clones, Type III clones were obtained from adult ICR, transgenic or mutant mice, or biopsy specimens from human temporal lobe, or from the adult human brain, or from dead animals with long post mortem intervals when the animals were kept at 4°C. The brain source was dissociated as described in Example 1 and Example 2 above, and the cells were grown in the suspension culture described above either without contact inhibiting factors, or, more often with a contact-inhibiting factor such as mercaptoethanol. The basic media for culturing type III cells was the same

as that used for culturing type II cells. Namely, the media comprised insulin (5 μg/mL), putrescine (100 μM), progesterone (20 μm), sodium selenite (30 μM), pituitary extract (20 μg/mL), transferring (100 μg/mL), 10 μg/mL basic fibroblast growth factor (bFGF), 10 ng/mL epidermal growth factor (EGF), and 5% fetal calf serum (FCS) in DMEM/F12 media. Cells were fed every 3-4 days by centrifugation and resuspension in fresh medium. After removal of the contact limiting factor, both type II and type III clones were apparent after 5-7 days. The type II clones eventually evolved into type III clones upon continued culturing in the absence of contact limiting factors.

Simply removing the contact inhibiting factors encourages differentiation by encouraging cell-cell contact. However, differentiation of type III clones into neurons or glia is also encouraged by other additional factors, including the growth factors like β-fibroblast growth factor, epidermal growth factor, or factors that are contained within pituitary extract present in the basic type III culture media. Other growth factors such as brain-derived neurotrophic factor (BDNF), glial derived neurotrphic factor (GDNF), NT3, and ciliary neurotrophic factor (CNTF) may also encourage differentiation of the stem/precursor cells.

The following chart summarizes the various methods to obtain the different stem/precursor cell types of the instant invention:

Steps	Type I clones	Type II clones	TYPE III clones	
Brain dissociation	+	+	+	+
Grow in suspension Culture	+	+	+	+
Add contact inhibiting factor (for ≤ 2 weeks)	+	+	+	+
Remove contact inhibiting factor		+	+	
Culture or plate on plastic/ laminin coated substrate			+	+

5.4 EXAMPLE 4 - FIXING OF CULTURES FOR STAINING OR ANTIBODY TESTING

Cultures are fixed in one of two ways depending upon the cultivation paradigm. Adherent clones (cultivated on plastic or laminin-coated plastic) are washed three times with room temperature Dulbecco's PBS (phosphate buffered saline) and then fixed either

with 10% acetic acid in pure ethanol at -20°C, or ice cold 4% paraformaidehyde in PBS. After ¾ to 1 hour, the fixative was removed and the cultures were washed three times with PBS.

Clones cultivated as suspension cultures were collected in a 15-ml plastic tube and centrifuged to form a pellet. Culture medium was aspirated and fixative (described above) was added to the pellet. Clones were then triturated and kept in fixative for ¾ to 1 hour, after which time the cells were again centrifuged and washed in PBS. Finally, the pellet was resuspended in a small volume of fresh PBS, and small aliquots of cells were placed on polylysine-coated coverslips and allowed to dry before the application of cellular stains or antibodies.

5.5 Example 5 - Preparation Of Cultures For Ultrastructural Analysis

Clones grown in suspension cultures were prepared for ultrastructural analysis by fixation in sodium cacocodylate buffer comprising 2% glutaraldehyde, 2% paraformaldehyde, 0.5% acroline, and 5% sucrose. The fixative was warmed to 37°C and gradually added to cultures until the fixative-to-medium ratio was 1:1. Cells were then collected in 15-ml tubes and centrifuged to form a pellet which was then covered with 100% fixative for several hours before processing with standard embedding, staining, and sectioning protocols. Samples were viewed on a JEOL 2000 electron microscope.

EM of type II clones revealed rings of small, tightly apposed cells that often surround a core of flocculent, non-cellular material (FIG. 3B) having many of the characteristics of extracellular matrix. The type II cell has many organelles, including endoplasmic reticalum, Golgi apparatus, dense bodies, and mitochondria.

EM of type III clones (see, FIG. 3D) revealed cells that appear to be more differentiated than type II cells, as their cytoplasm is less dense than type IIs and their organelles appear to be more developed. FIG. 7 shows EM of a clone from a transgenic mouse (tenascin knockout mouse) and a sphere (clone) from an adult human temporal lobectomy specimen.

5.6 EXAMPLE 6 - CULTURING OF CELLS IN METHYLEELLULOSE FOR OBSERVATION OF SINGLE CLONES

Methylcellulose (StemCell Technologies, Vancouver, B.C.) was dissolved in medium (at a concentration of 1.6%, and Dulbecco/FI2 + N1 supplement medium was added, with an equal volume of brain cells suspension, to a final concentration of 0.8% methylcellulose (see, Worton *et al.*, 1969). Cells were fed every 2-3 days by the addition of small aliquots of medium without methylcellulose. Single clones were followed over time (FIG. 5), and observed to increase in size indicating cell proliferation and growth.

5.7 EXAMPLE 7 - TESTING CLONES FOR REACTIVITY TO CELL MARKERS

Standard immunofluorescence techniques were used with antibodies to polyclonal GFAP (Immunon), monoclonal β III-tubulin (Sigma), polyclonal L1, monoclonal nestin (Developmental Hybridoma Bank), and 04 (Chemicon). Cells were also labeled with propidium iodide (Sigma).

Type II clones are immunonegative for cell-specific markers, including GFAP, nestin, and TuJ1. These are considered early type II clones. However, after approximately 10 days *in vitro*, some cells of type II clones become immunopositive for nestin but remain immunonegative for GFAP and TuJ1. Type III clones exhibit cell phenotype markers of more differentiated cells being immunopositive for nestin, GFAP, L1, and TuJ1. This staining pattern shows the continuum of evolution of type II clones from early (immunonegative) to late (selectively immunopositive) and eventually to type III clones (fully immunopositive) (FIG. 5).

Other techniques can be used to distinguish type II and type III clones. For example, rtPCR can be used to reveal genes that are different between type II and type III clones. Not only will this further characterize the type II and type III clones, but it will also reveal novel genes in each clone type, as well as identify genes involved in different developmental stages of neuronal precursor cells.

5.8 EXAMPLE 8 - USING BRAINS FROM DEAD MAMMALS AS A SOURCE OF STEM/PRECURSOR CELLS

Using exactly the same procedures as outlined for generating type I, II and III spheres from acutely dissociated brain tissue, it is also possible to generate type I, II and III clones from brain tissue of animals with significantly long postmortem intervals (FIG. 7B). Thus far, dissociated brain from adult mice with postmortem intervals from 16-24 hours have yielded normal type I, type II and type III spheres. Pilot studies have indicated that it might be possible to harvest brain tissue (if stored at 4°C) from animals or human cadavers with postmortem intervals of up to 2-5 days. These findings have implications for "banking" of mature human brain tissue specimens for experimentation as well as transplantation for traumatic injuries and neurological disease.

5.9 EXAMPLE 9 - LIGHT AND ELECTRON MICROSCOPY OF CLONES FROM ADULT HUMAN BRAIN

Human brain also generates type II and III clones, as described for the adult rodent. Dissociating biopsy specimens from the adult human temporal lobe may be used to generate type II and II spheres using the same culture methods as described for isolating type II and type II spheres from rodent brain tissue. In addition, similar to the adult rodent, human brain may also generate type I clones. FIG. 7D shows human spheres at the light and electron microscopic level that have many of the same cytological feature as described in rodent spheres.

5.10 EXAMPLE 10 - TRANSPLANTATION (GRAFTING) OF TYPE I OR II CLONES FROM ROSA-26 ADULT MICE

Type II or III clones, generated as described in Examples 2 and 3 (see, above) from adult ROSA-26 transgenic mice (Friedrich *et al.*, 1991) were aspirated, with media, into a Hamilton microsyringe with an attached 31 gauge needle using a video stereomicroscope set-up. 1 µl was slowly injected into the adult ICR mouse striatum. For stereotaxic coordinates, as well as details for the histochemical detection of β-galactosidase activity (Gates *et al.*, 1996). Type II and III clones were also found to exhibit X-gal labeling *in vitro* (see, FIG. 6). Survival times of 7-14 days were observed

thus far, with a 10-day survival shown in FIG. 6. It is possible that the transplants can survive longer times, simply carrying the experiment out for a longer time point will determine that. Preliminary studies also indicate that grafted type II and III spheres from adult human brains give rise to cells that survive in the mature brains of immunocompromised mice.

In addition, the transplants can differentiate into different types of resident neuronal populations. For example, some of the transplanted spheres from β-galactosidase-positive mice differentiate into astrocytes in the host ICR (non-β-galactosidase-positive brain); some transplanted spheres differentiate into neurons. Immunocytochemistry with GFAP revealed the presence of labeled astrocytes and non-labeled larger cells that are presumed to be neurons. Furthermore, *in vitro* cultures using feeder cells from different parts of the body (*i.e.* endothelial cells, lung endothelial cells, or kidney cells) change the gross morphology of the spheres generated. Both of these *in vitro*, and *in vivo* experiments indicate that the environment into which the spheres are transplanted contributes to the type of cells into which they will differentiate. It may therefore be possible to direct the phenotype of the precursor/stem cells using cell feeder layers from specific tissues, as well as other molecular priming approaches, and genetic manipulations (*e.g.* transfections, or viral infections).

The transplant studies described above were performed in "normal" brain circuitries. Such studies indicate that type II and III spheres give rise to glia and neuronal cells that can survive the grafting procedure. Similar experiments can be done in compromised circuitries to determine whether or not compromised circuitries result in any lineage restriction or allow further differentiation.

5.11 EXAMPLE 11 - GENERATION OF MONOLAYER CULTURES

Astrocyte monolayers were derived from the cerebral cortex, cerebellum, and spinal cords of early postnatal (postnatal day 1-10), late postnatal (postnatal day 11-20), and adult (>30 days) ICR or *Gtv-a* transgenic mice. Mice were deeply anesthetized and decapitated. Cerebral cortex, cerebellum, spinal cord, and SEZ were dissected and processed separately to generate astrocyte monolayers. The cerebral cortex dissection was performed in such a way as to exclude all cells of the ventricular or subependymal

region. This was accomplished by first blocking the rostral forebrain in the coronal plane, and then shaving superficial slices of the cerebrum tangential to the pial surface. Primary cultures were generated by mincing the tissue with a razor blade, incubating in trypsin, and triturating with fire-polished glass pipettes. The resulting cell suspension was seeded in a flask in DMEM/F12 medium containing 10% FCS, and allowed to grow to confluence.

5.12 EXAMPLE 12 - GENERATION OF NEUROSPHERES FROM ASTROCYTE MONOLAYERS

Confluent astrocyte monolayers were trypsinized, pelleted, and resuspended in serum-free 2 × N2 medium. Cells were counted with a hemacytometer, and live cells were determined by trypan blue exclusion. DF medium containing 2% methylcellulose was then added 1:1 to the cells, which were then plated a density of 1000 cells/cm2 in 6-well plates coated with the antiadhesive poly (2-hydroxyethyl methacrylate) as previously described (Kukekov *et al.*, 1997). Cultures were supplemented with 10ng/mL of EGF and bFGF every 2-3 days.

BrdU labeling

In order to label proliferating cells, BrdU (10μg/mL) was added to suspension cultures for 24 hours prior to removal and plating of neurospheres. Detection of BrdU labeled cells followed these steps: fixation of neurospheres with paraformaldehyde as described below; incubation in 1:12X SSC:formamide for 2 hrs. at 65°C); incubation in 2N HCl for 30 min. at 37°C; equilibration in borate buffer for 10 min. at room temperature. Finally, immunofluorescence with monoclonal anti-BrdU was performed as described below.

5.13 EXAMPLE 13 - IMMUNOCYTOCHEMICAL STAINING OF CLONES

Clones were removed from individual microwells, or methylcellulose suspension culture, and placed into a drop of N2 medium containing 1% FCS on glass coverslips that had been coated sequentially with poly-l-ornithine (10μg/mL) and laminin (5μg/mL). One to two days after plating on coverslips, cells were fixed with 4% paraformaldehyde in PBS for 20 min., and processed, as previously described (Johansson *et al.*, 1999;

Kukekov *et al.*, 1997) for immunocytochemistry with antibodies against the following antigens: glial fibrillary acidic protein (GFAP), an astrocyte-specific intermediate filament protein (Immunon); vimentin and nestin, cytoskeletal markers of immature astrocytes, and neural stem cells (Developmental Hybridoma Bank); β III-tubulin, a neuron-specific intermediate filament protein (Promega or Sigma); L1, a cell adhesion molecule associated with neurons; MAP-2, a microtubule protein associated with neurons; A2B5, a surface protein associated with O2A progenitors capable of giving rise to either astrocytes or oligodendrocytes (Boehringer Mannheim). Some cells were counterstained with propidium iodide before viewing. FITC and AMCA secondary antibodies were used to visualize the primary antibody binding sites.

5.14 EXAMPLE 14 - PHENOTYPE OF MONOLAYER CULTURES

In order to examine the potential for astrocytes to display NSC characteristics, we first generated monolayer cultures of astrocytes from cerebral cortex, cerebellum, spinal cord, and SEZ from mice ranging in age from late embryonic to adult. Because of the close juxtaposition of the cerebellum and spinal cord to the ventricular system, we could not be certain of excluding all periventricular cells from the dissociation of these tissues. However, cerebral cortex dissociations followed a careful dissection procedure whereby, with the aid of a dissecting microscope, only the superficial cortical surface was isolated, excluding the possibility of contamination by ependymal or subependymal cells.

The primary fraction of cells used to generate astrocyte monolayers initially contains cells that are immunopositive for a variety of phenotypic antigens, including the oligodendrocyte membrane protein O4, and neuron specific β III-tubulin (data not shown); however, after 7-10 days *in vitro*, plated cells form a confluent monolayer (FIG. 8A), and these markers fail to label any cells. At this stage, a majority of cells in the monolayer are immunopositive for the astrocyte-specific intermediate filament protein GFAP (FIG. 8B); however, since some of the cells in the monolayer are weakly labeled by, or do not express this marker we also show that an even greater percentage (95%-100%) of the monolayer cells are immunopositive for other markers of immature and reactive astrocytes, including vimentin (FIG. 8C), as well as nestin (not shown) and S100β (FIG. 8C, inset). Within the primary fraction, the only other immunologically

identifiable cells are microglia which express the antigen Mac-1 (FIG. 8A, lower right inset). The percentage of Mac-1 positive cells present in the primary fraction typically ranges from 1-5%, and Mac-1 immunostaining is essentially abolished by passaging, leaving only cells with astrocytic morphology and antigen expression. Monolayer cultures are immunonegative for collagen types I and IV, and Von Willebrand's Factor, indicating that there is no detectable contamination either by fibroblasts or endothelial cells.

5.15 EXAMPLE 15 - NEUROSPHERES GENERATED FROM ASTROCYTE MONOLAYERS ARE PLEURIPOTENT

When astrocyte monolayers are detached and grown as suspension cultures in the presence of EGF and Bfgf (Kukekov *et al.*, 1997) between 1 and 10% of the cells generate spherical clones (FIG. 8A, upper left inset). We believe that these structures are composed of clonally-related cells, and do not represent cellular aggregates for the following reasons: first, we plated dissociated astrocytes in semi-solid methylcellulose medium that hinders cell motility; second, they have a sharp, continuous outer border unlike that seen with cellular aggregates; third, we do not see these structures when growth factors are omitted; fourth, the addition of BrdU to the culture medium can label virtually all of the cells within a sphere, indicating that each cell is newly-generated (FIG. 9A, upper left inset); and fifth, it is possible to generate a similar structures when single astrocytes are cultured individually in microwells (FIG. 8A, upper right inset). When removed from suspension culture and plated onto a favorable substrate (polyornithine/laminin coated glass), astrocyte-derived spheres readily attach, and the cells at the bottom begin to migrate, differentiate, and send out processes (FIG. 8B, inset and FIG. 9). The largest percentage of cells derived from the sphere are immunopositive for the astrocyte-specific filament protein GFAP (FIG. 9A and inset); however, many cells are observed to be immunopositive for the oligodendrocyte-type 2 astrocyte progenitor cell (O-2A) marker A2B5 (FIG. 9A, lower inset), as well as the neuron-specific markers β III-tubulin, L1 and MAP-2 (Fig. 9B-3).

5.16 EXAMPLE 16 - USE OF GTV-A MICE TO CONFIRM ASTROCYTIC IDENTITY OF NEUROSPHERE-FORMING CELLS

INFECTION OF GFAP-TVA ASTROCYTES, AND ALKALINE PHOSPHATASE HISTOCHEMISTRY

Astrocyte monolayers were prepared as described above from the cerebral cortices of P2 *Gtv-a* transgenic mice. Avian leukosisvirus containing the *Lac-Z* insert or alkaline phosphatase (AP); enzyme histochemistry, detected with an AP detection kit as described by the vendor, Vector Labs) as a marker, were produced by chicken fibroblasts (FIG. 10A, upper inset), and infection of astrocytes was accomplished by continuously incubating *Gtv-a* astrocytes in medium conditioned by the fibroblasts. Regarding the sensitivity of virus internalization, typical infection rates in the astrocyte monolayers are 1-5%, with the best infection rate being 16%. There is very little chance for successfully infecting a cell with immunologically undetectable levels of GFAP, and all of the cells in the monolayer cultures express one or another astrocyte phenotypic marker (*i.e.* GFAP, nestin, vimentin, or S100B), and with the addition of small numbers of Mac-1 labeled microglial cells in the monolayer cultures, the totals equal 100% of propidium iodide-counterstained cells.

We utilized the *Gtv-a* transgenic mouse to selectively infect astrocytes with a virus, and subsequently showed that neurosphere-derived neurons also contained the virus. This transgenic mouse was constructed such that the gene encoding the TVA receptor is under control of the GFAP promoter (GFAP is an astrocyte-specific intermediate filament protein). TVA expression is required for infection with avian leukosis virus. Mouse cells not expressing GFAP are non-infectable with the avian leukosis virus. Monolayers of astrocytes from the *Gtv-a* mouse were grown in the presence of an avian leukosis virus containing the alkaline phosphatase (AP) reporter gene in order to visualize infected cells. When infected cells divide, the virus is incorporated into the genome, and is therefore passed on the all progeny. Expression of AP is not under the control of the GFAP promoter, but rather is under control of the viral LTR gene which results in constitutive expression of AP; this means that the progeny of an infected astrocyte will express AP even if the GFAP promoter is never active in this cell. When neurospheres were derived from monolayers of *Gtv-a* astrocytes (containing

variable numbers of infected cells), some clones were AP-positive. Combining AP histochemistry with immunocytochemistry for neuronal antigens (*i.e.* β III-tubulin) showed that neurons expressed AP, and therefore must be the progeny of an infected astrocyte.

5.17 Example 17 - Age and Regional Effects on the Ability of Astrocyte Monolayers to produce Spherical Clones

The ability of astrocyte monolayers to produce spherical clones is discretely age- and region-related (FIG. 11). When we compare the spatiotemporal characteristics of the tissues from which the astrocyte monolayers where derived, it is apparent that neurospheres are generated from astrocyte monolayers derived from cerebral cortex, cerebellum and spinal cord only when these monolayers originate from animals younger than postnatal day 11. In contrast, SEZ-derived astrocytes continue to produce clones even when generated from animals older than P11. Additionally, astrocyte monolayers maintain neurosphere-generating ability even after five passages, but there appears to be a gradual reduction in neurosphere yield with increasing passage.

5.18 Example 18 - Fibroblasts and Microglia

Fibroblasts were obtained from mouse muscle. First postnatal week mice were anesthetized and decapitated. The limbs and torso were stripped of skin, and the thoracic, abdominal, and pelvic viscera were removed. The remaining carcass was minced with a razor blade, incubated in trypsin, and tirturated with a fire-polished Pasteur pipette. The resulting cell suspension was seeded in tissue culture flasks as described above to yield a highly enriched culture of fibroblasts. Microglia cultures were generated from primary monolayers derived from brain as described above for astrocyte cultures. After the primary brain dissociate reached confluence, 20% colony stimulating factor (CSF),and dextran microbeads (Cytodex, Pharmacia) were added to the culture. After approximately one week, the beads were removed and lightly vortexed to removed adherent cells. The beads were allowed to sediment, and the resulting cell suspension was aspirated, centrifuged, and replated in a flask containing DMEM/F12 with 20% CSF,

and 10% FCS. This protocol yielded a highly enriched culture of microglia containing few cells with astrocyte morphology.

In order to rule-out that cells other than astrocytes are responsible for generating the neurospheres, we tested the ability of the two likeliest contaminating cells—fibroblasts and microglia—to form neurospheres in our suspension culture paradigm. Neither purified cultures of mouse microglia nor fibroblasts formed neurospheres under these conditions. These two control experiments strongly suggest that the cells giving rise to multipotent neurospheres are, in fact, astrocytes; FIG. 12 shows fibroblast monolayers, derived from mouse muscle, form spherical clones when replated in suspension culture supplemented with EGF and FGF2 (arrowheads in FIG. 12A). When attached and allowed to differentiate, fibroblast-derived clones give rise only to cells with fibroblast-like morphology (FIG. 12B), and these cells are never immunopositive for neural-specific antigens. Monolayers of microglia (FIG. 12C) fail to generate spherical clones when replated in suspension culture supplemented with EGF and FGF1.

5.19 Example 19 - Pluripotent Neurospheres from Anaplastic Astrocytoma Cells

When stem/progenitor cells are harvested and cultured from an adult human brain anaplastic astrocytoma (this example, but also from astrocytomas and glioblastomas) both astrocytes (immunofluorescence for astrocytic glial fibrillary acidic, GFAP, protein) and neurons (immunofluorescence for neuronal beta III tubulin) arise from multipotent neurospheres in suspension culture (with semi-solid, i.e. methylcellulose-containing media) and when plated on laminin or poly-L-ornithine. FIG. 13A. Two attached clones from this anaplastic astrocytoma seem to merge, with neurons and astrocytes within and surrounding the clones. FIG. 13B. Two clones immunostained for just neuronal β III-tubulin shows many neurons that arise from such glial tumors. FIG. 13C. Two different clones, from the same tumor, again stained for the same astrocytic and neuronal markers. FIG. 13D. A single, β III-tubulin positive neuron that is outside of the attached clone, and exhibits unusual somatodendritic morphology. FIG. 13E. Higher magnification of neurons and astrocytes with apparently normal morphologies. FIG. 13F and FIG. 13G. The same field of cells immunostained for GFAP (FIG. 13F) and neuronal β III-tubulin

(FIG. 13G). It can be seen that there are single cells expressing both neuronal and glial antigens, which is not normally seen within clones derived from non-tumor stem/progenitor cells.

It will be apparent to those skilled in the art that various modifications and variations can be made in the methods of the present invention without departing from the spirit or scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

6.0 REFERENCES

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WHAT IS CLAIMED IS:

1. A method of producing multipotent stem/progenitor cells from mammalian brain tissue, comprising culturing dissociated mammalian brain tissue for a period of time sufficient to form an astrocyte monolayer and dissociating astrocyte cells from said monolayer to obtain multipotent stem/progenitor cells wherein at least a fraction of said cells are clonogenic.

2. The method of claim 1 wherein the mammalian brain tissue is from cerebral cortex, cerebellum, spinal cord or subependymal zone (SEZ) tissue.

3. The method of claim 2 wherein the brain tissue is adult human brain tissue.

4. The method of claim 3 wherein the brain tissue is up to about five days post-mortem mammalian brain tissue.

5. The method of claim 3 wherein the human brain tissue is a brain tumor.

6. The method of claim 5 wherein the brain tumor is an astrocytoma or a glioblastoma.

7. The method of claim 6 wherein the human brain tumor is an anaplastic astrocytoma.

8. The method of claim 1 wherein the cultured multipotent stem/progenitor cells are obtained from a mammalian astrocyte monolayer transduced with a DNA or a DNA comprised within a suitable transfecting vector..

9. The method of claim 1 further comprising culturing in the presence of cell contact inhibitors to allow formation of an astrocyte stem/progenitor cell-derived neurosphere from said clonogenic cells.

10. The method of claim 9 further comprising removing cell-cell contact inhibitors from the culture media and culturing under conditions that promote differentiation of said astrocyte-derived cells into neurons.

11. The method of claim 1 further comprising preparing a single cell suspension from said dissociated astrocyte cells and culturing said single cell suspension in the presence of at least one cell-contact inhibiting compound and at least one growth factor to provide a cell population comprising at least in part multipotent stem/progenitor astrocyte cells.

12. The method of claim 11 wherein the cell-contact inhibiting compound is β-mercaptoethanol, methylcellulose or poly 2-hydroxyethyl methacrylate.

13. The method of claim 11 wherein the growth factor is selected from the group consisting of basic fibroblast growth factor, epidermal growth factor, brain derived neurotrophic factor, glial derived neurotrophic factor, neurotrophin-3 and ciliary neurotrophic factor.

14. The method of claim 11 wherein at least a portion of said cell population is clonogenic.

15. The method of claim 11 wherein said cell population comprises neurons and glia.

16. The method of claim 11 wherein the cell population comprises clones (neurospheres).

17. The method of claim 16 further comprising dissociating the clones to form a cell suspension comprising multipotent astrocyte stem/progenitor cells and transplanting said suspension into a brain of a mammal wherein at least a portion of said cells differentiate into neurons.

18. The method of claim 17 wherein at least a portion of said cells differentiate into glia.

19. The method of claim 11 further comprising differentiating the multipotent astrocyte stem/progenitor cells according to the steps:

removing cell-contact inhibiting substances from the culturing media; and,

culturing the multipotent stem/progenitor cells in the presence of growth factors selected from the group consisting of basic fibroblast growth factor, epidermal growth factor, brain derived neurotrophic factor, glial derived neurotrophic factor, neurotrophin-3 and ciliary neurotrophic factor to produce differentiated cells;

wherein the multipotent astrocyte stem/progenitor cells differentiate into neurons, astrocytes and oligodendrocytes.

20. The method of claim 11 further comprising removing cell contact inhibitors from the culture media and culturing the multipotent stem/progenitor cells with feeder cells from selected tissues to differentiate said cells into a phenotype determined by phenotype characteristics of said feeder cells.

21. A multipotent stem/progenitor cell isolated from mammalian brain tissue obtained post mortem up to five days or from biopsy of central nervous system tissue in a human up to 2 years of age from the subependymal zone of any age human wherein said cell is prepared from an astrocyte monolayer by the method of claim 1.

22. A composition comprising a suitable vehicle and the astrocyte cells of claim 21.

23. The composition of claim 22 wherein the vehicle is a pharmaceutically acceptable vehicle.

24. The composition of claim 22 wherein the multipotent stem/progenitor cells are clonogenic and are prepared from a tumor tissue.

25. The composition of claim 24 wherein the tumor tissue comprises human glioma cells.

26. The composition of claim 24 wherein the tumor clonogenic multipotent stem/progenitor cells are morphologically distinct from multipotent stem/progenitor cells generated from normal neural tissue.

27. An isolated multipotent stem/progenitor brain cell clone (neurosphere) characterized by the following properties:

continuous, sharply defined spherical outer border;
cultured from dissociated brain tissue;
immunonegative for cell-specific markers;
lacking ability to attach to a matrix surface; and,
comprising multipotent stem/progenitor cells (Type I).

28. The neurosphere of claim 27 wherein the surface is plastic, laminin/polyornithine coated surface, polylysine, or cell/tissue surface.

29. The neurosphere of claim 27 wherein the multipotent stem/progenitor cells differentiate into neural and glial cells when plated onto a suitable substrate.

30. The neurosphere of claim 27 wherein the detached astrocyte monolayer is obtained from mammalian brain tissue.

31. The neurosphere of claim 30 wherein the mammalian brain tissue is human brain tissue.

32. The neurosphere of claim 31 wherein the human brain tissue is from cerebral cortex, cerebellum, spinal cord or subependymal zone (SEZ).

33. The neurosphere of claim 31 wherein the human brain tissue is postnatal biopsy tissue from about day 1 up to about day 730.

34. The neurosphere of claim 31 wherein the human brain tissue is a sample obtained up to 5 days postmortem.

35. The neurosphere of claim 31 wherein human brain tissue is from adult brain subependymal zone.

36. The neurosphere of claim 35 wherein the adult brain tissue is from postmortem tissue up to about 5 days post mortem.

37. A kit comprising the composition of claim 22, instructions for preparing clonal populations of glia and neurons and, optionally, reagents and growth factors for culturing the cells.

FIG. 1A

FIG. 1B

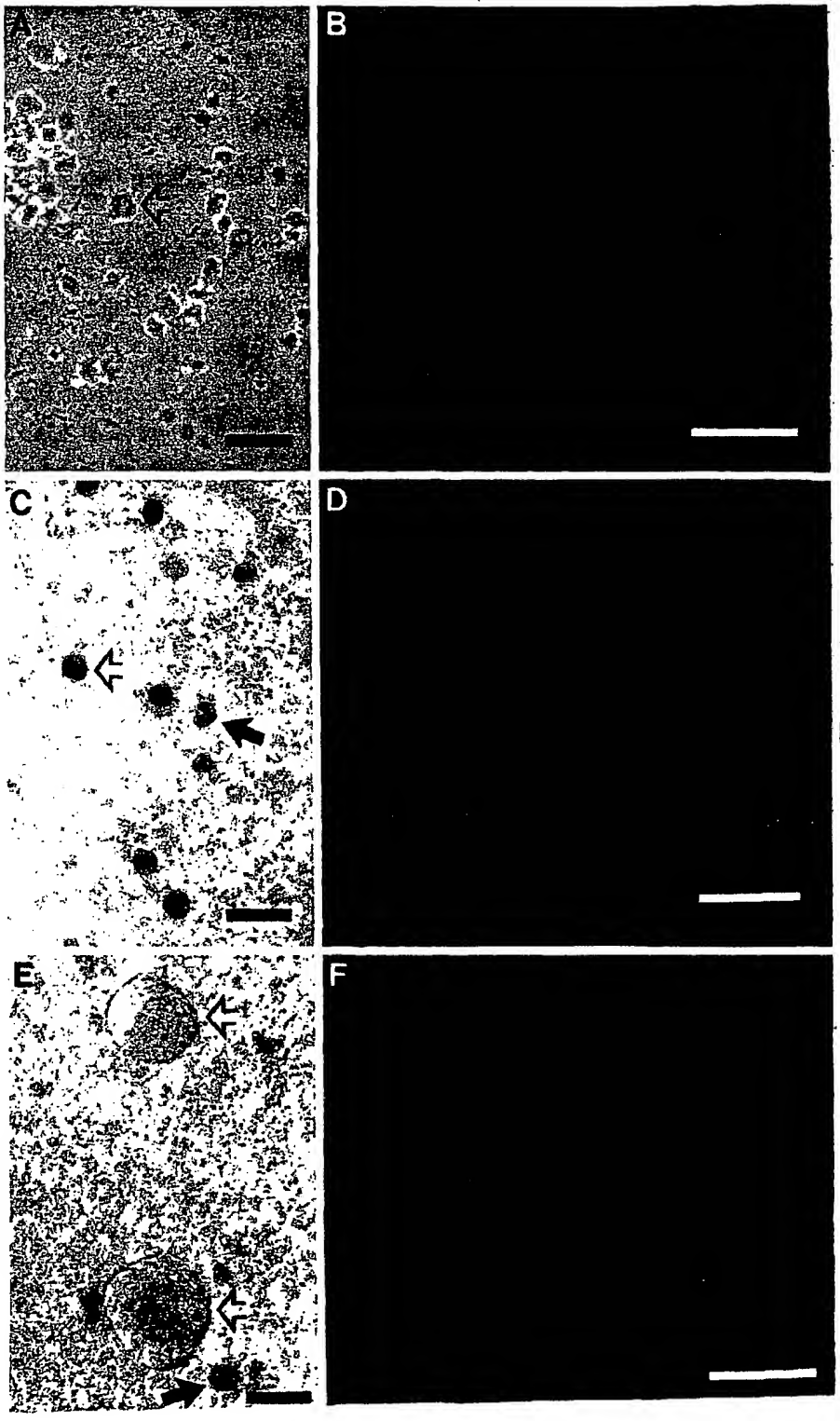


FIG. 1C

FIG. 1D

FIG. 1E

FIG. 1F

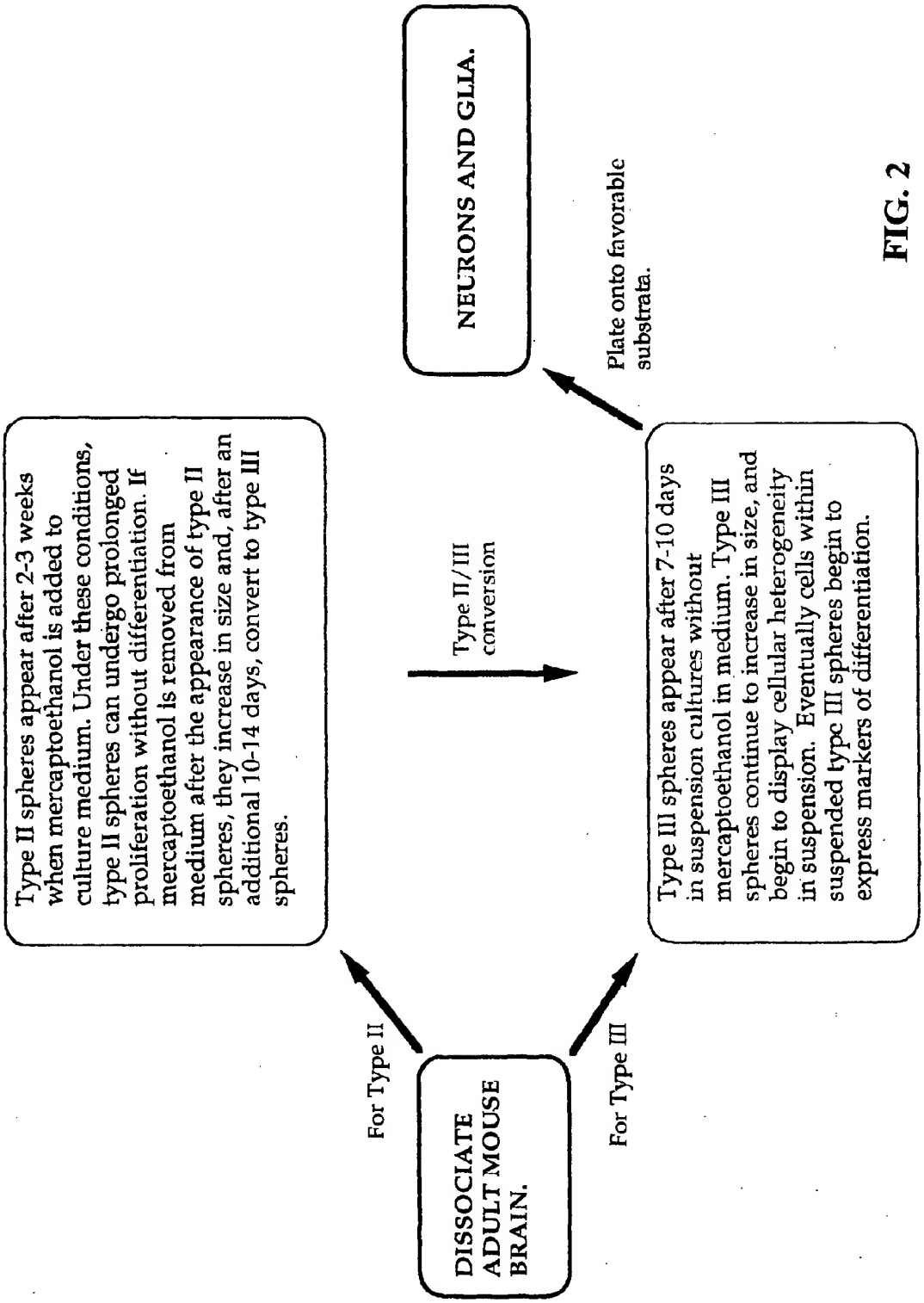

DISSOCIATE ADULT MOUSE BRAIN.
For Type II
For Type III
Type II spheres appear after 2-3 weeks when mercaptoethanol is added to culture medium. Under these conditions, type II spheres can undergo prolonged proliferation without differentiation. If mercaptoethanol is removed from medium after the appearance of type II spheres, they increase in size and, after an additional 10-14 days, convert to type III spheres.
Type II/III conversion
Type III spheres appear after 7-10 days in suspension cultures without mercaptoethanol in medium. Type III spheres continue to increase in size, and begin to display cellular heterogeneity in suspension. Eventually cells within suspended type III spheres begin to express markers of differentiation.
Plate onto favorable substrata.
NEURONS AND GLIA.

FIG. 2

FIG. 3A

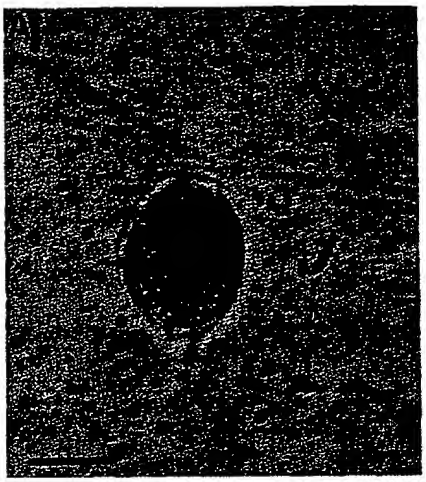
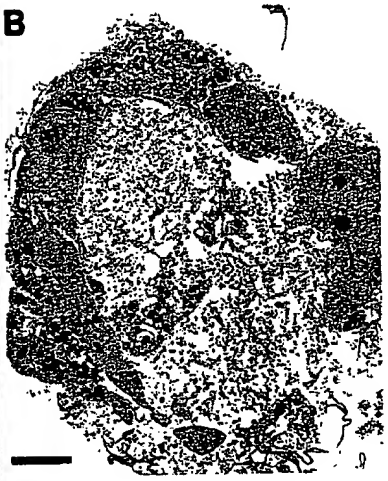


FIG. 3B



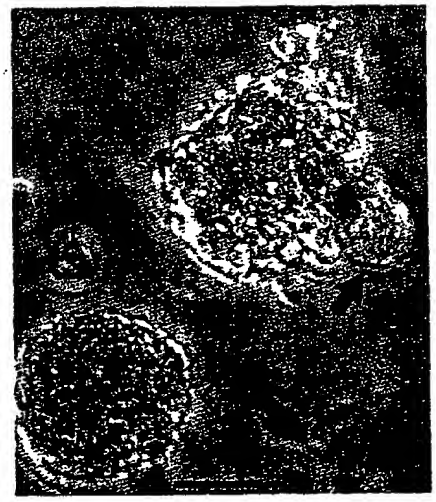


FIG. 3C

FIG. 3D

FIG. 4A FIG. 4B FIG. 4C

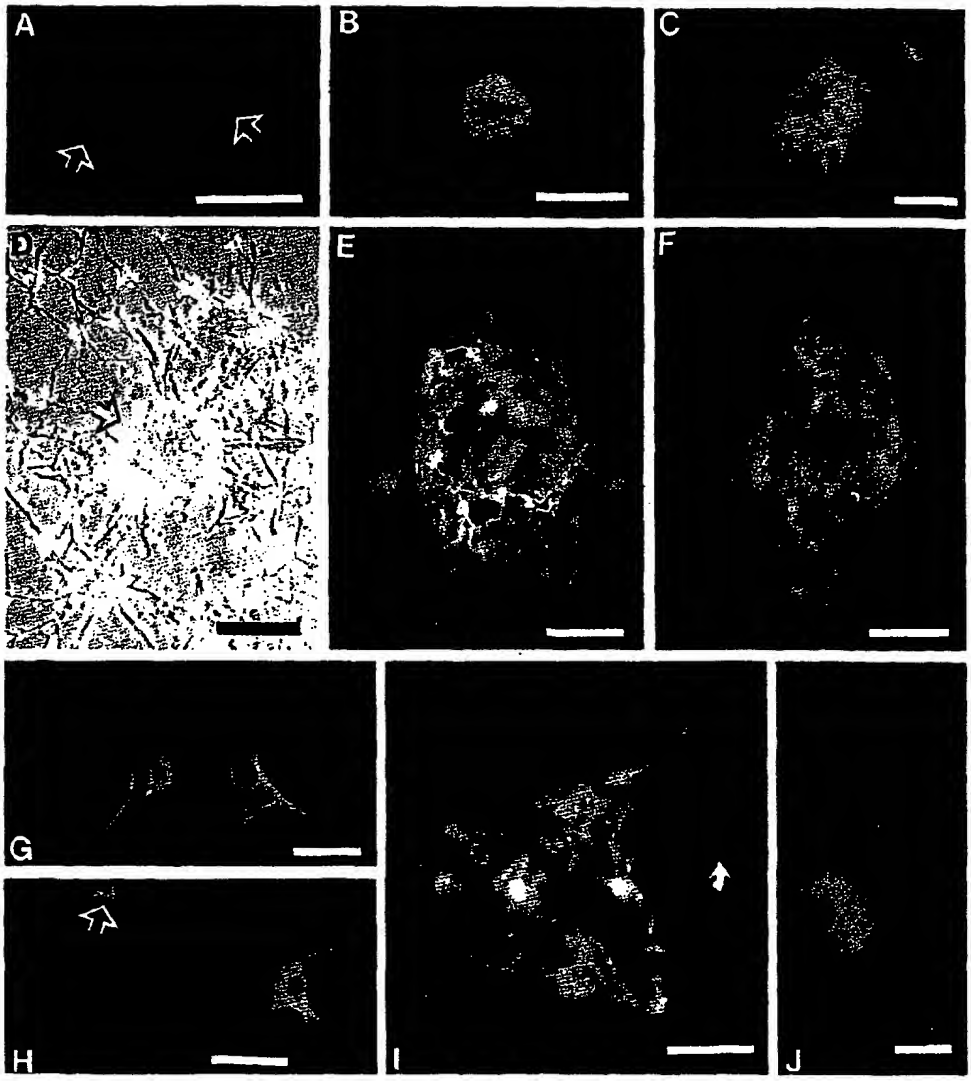


FIG. 4D FIG. 4F FIG. 4G

FIG. 4H FIG. 4I FIG. 4J

FIG. 5A

FIG. 5B

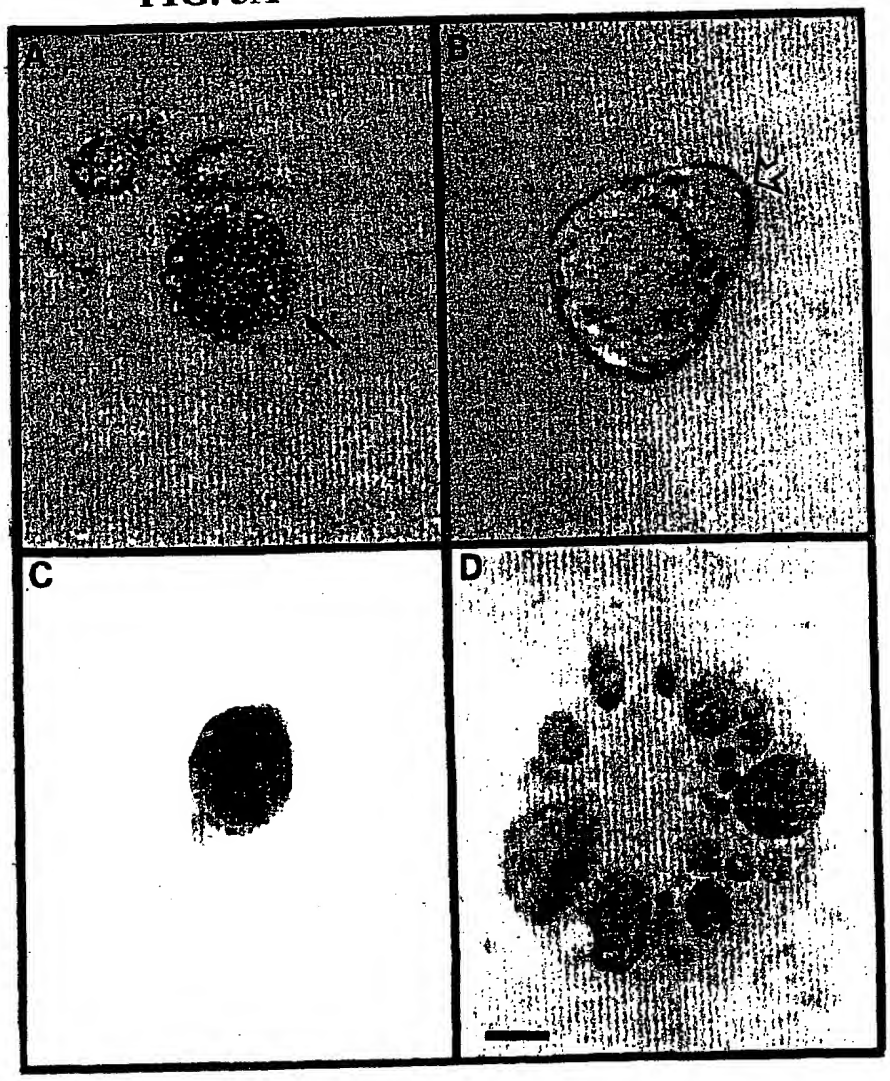


FIG. 5C

FIG. 5D

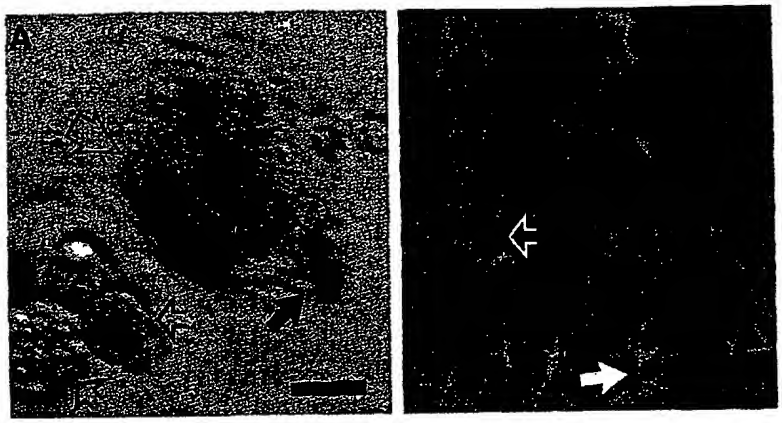


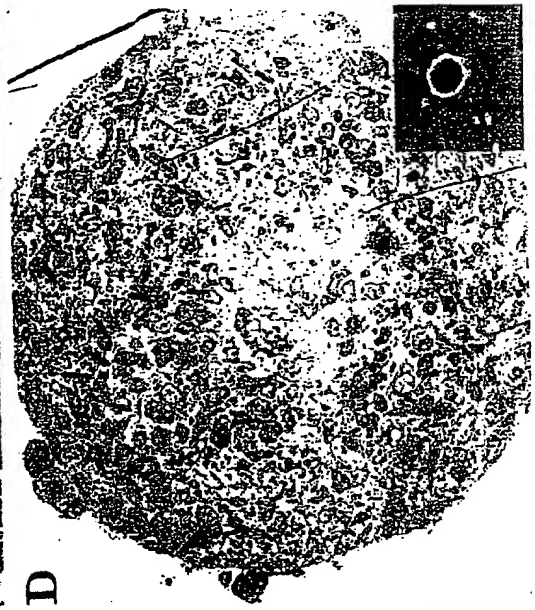
FIG. 6A

FIG. 6B

FIG. 7B

B

FIG. 7D



D

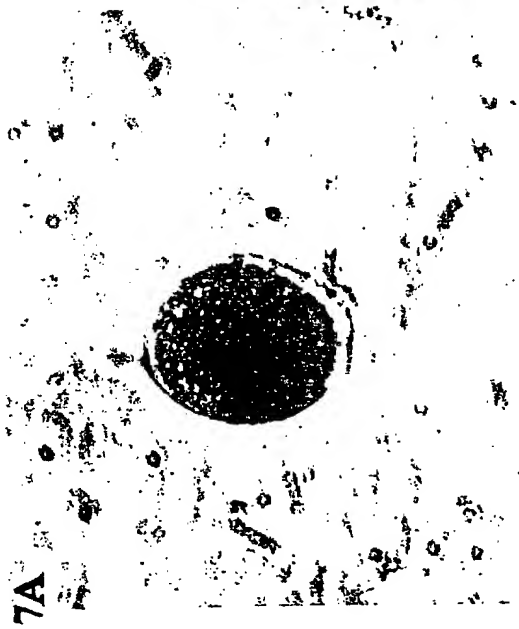


FIG. 7A

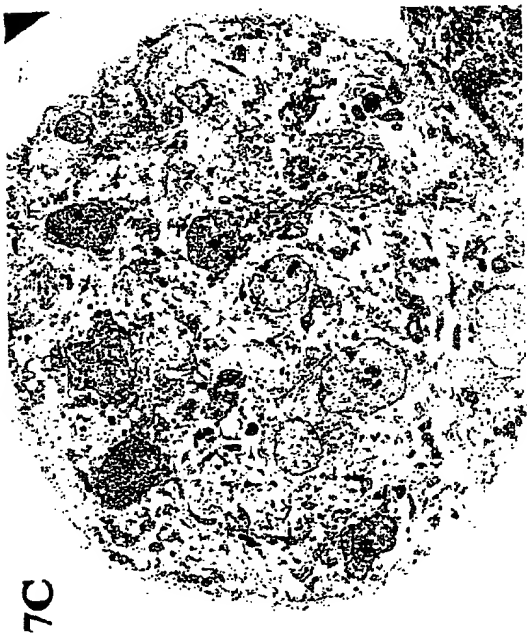


FIG. 7C

FIG. 8A

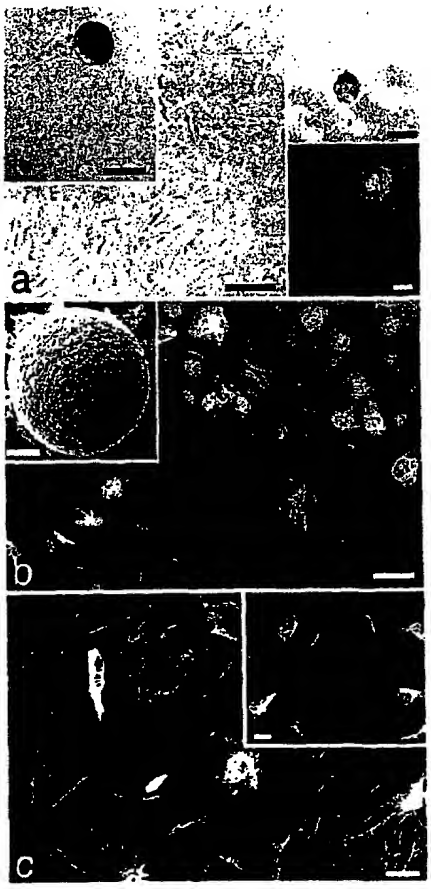


FIG. 8B

FIG. 8C

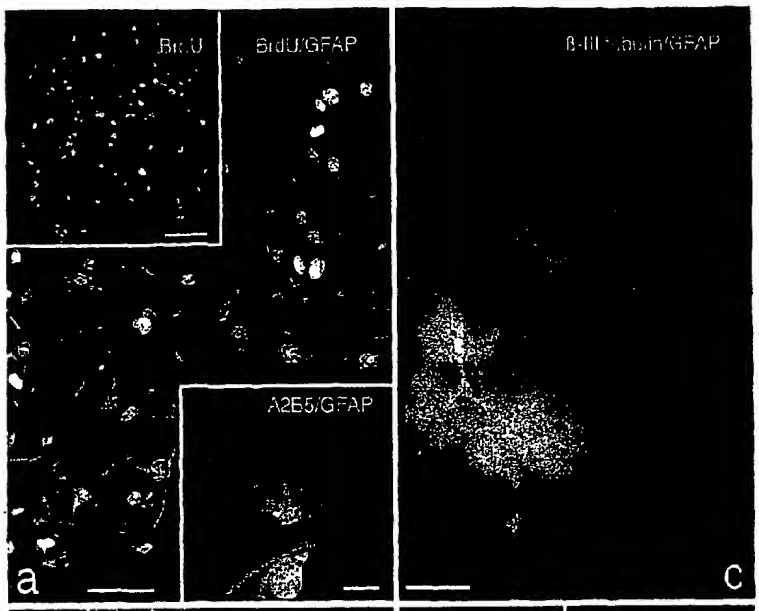


FIG. 9A

FIG. 9C

FIG. 9B

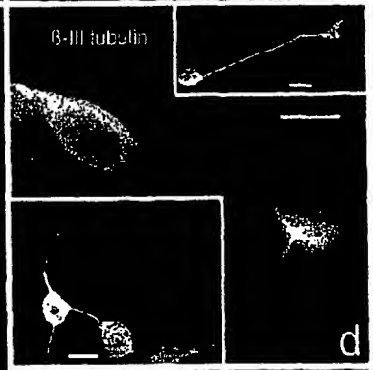


FIG. 9D

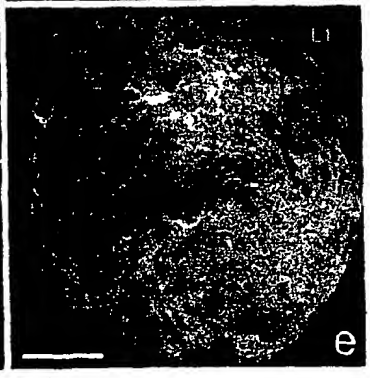


FIG. 9E

FIG. 10A

FIG. 10B

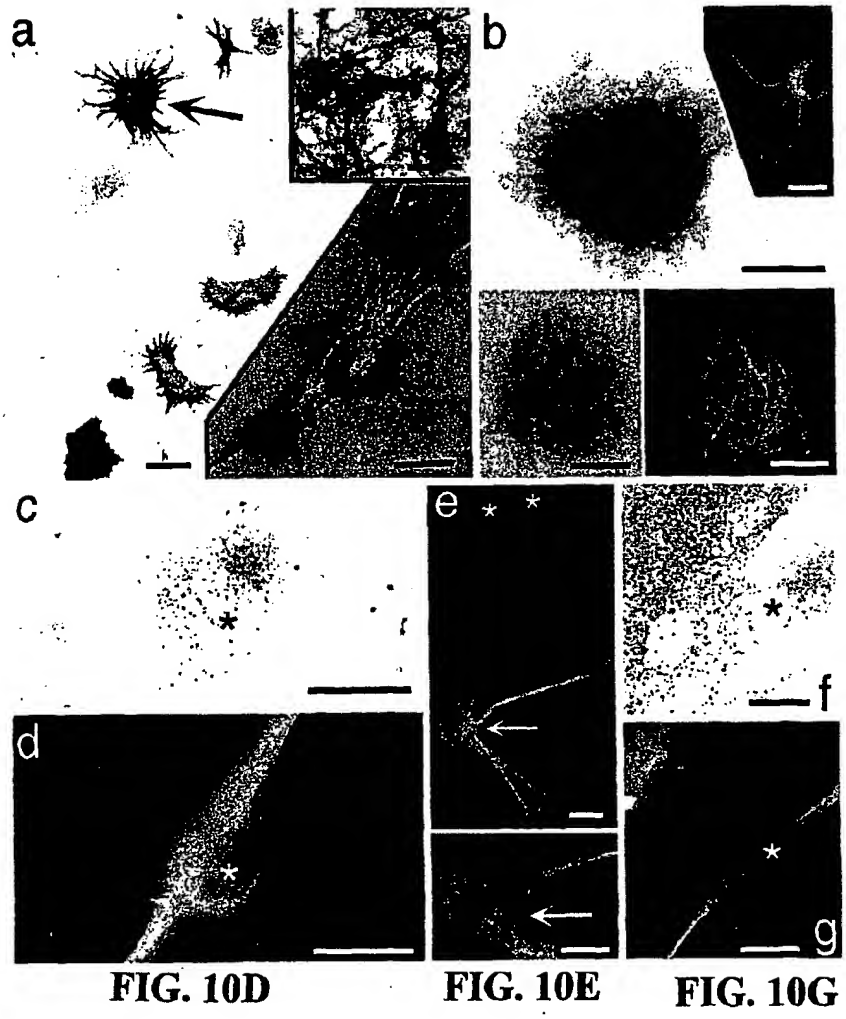


FIG. 10C

FIG. 10F

FIG. 10D

FIG. 10E

FIG. 10G

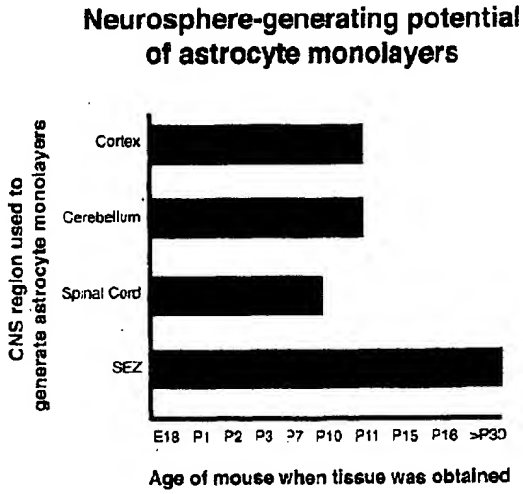

Neurosphere-generating potential of astrocyte monolayers
CNS region used to generate astrocyte monolayers
Cortex
Cerebellum
Spinal Cord
SEZ
E18 P1 P2 P3 P7 P10 P11 P15 P16 >P30
Age of mouse when tissue was obtained

FIG. 11

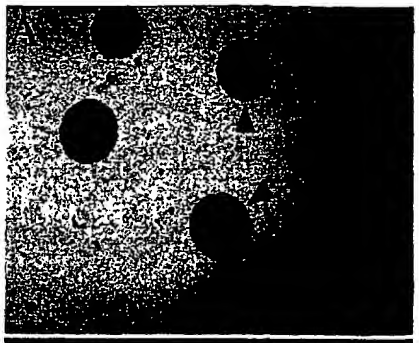


FIG. 12A

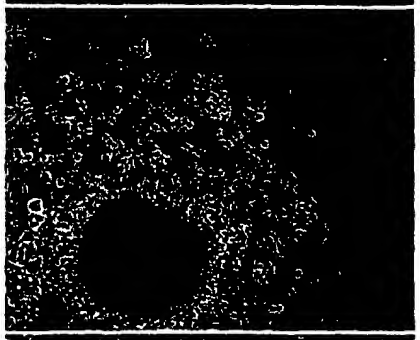


FIG. 12B

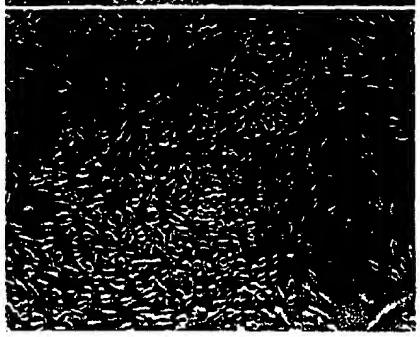


FIG. 12C

FIG. 13A

FIG. 13B

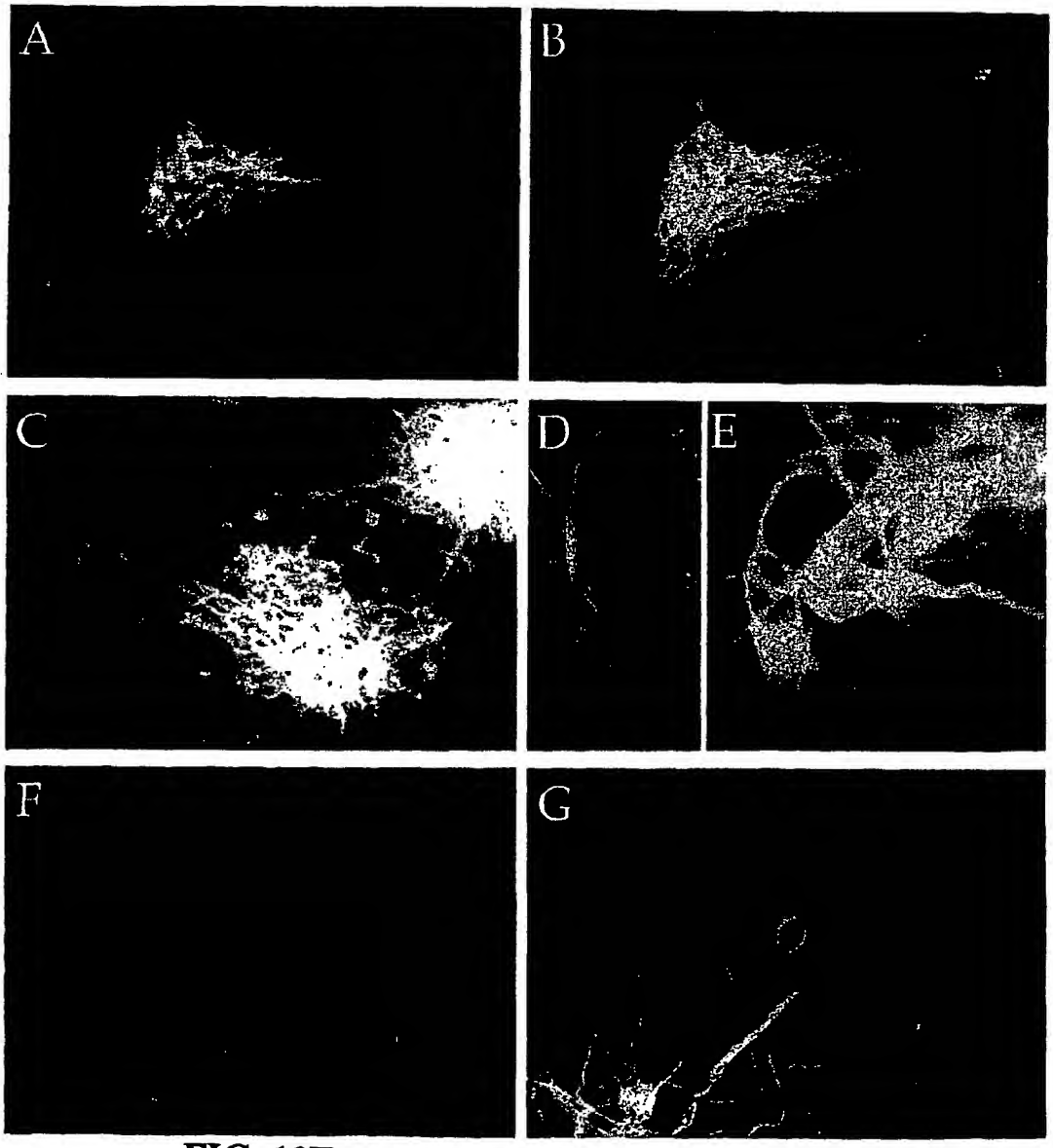


FIG. 13F

FIG. 13G